Sex determination and differentiation in the brown alga Ectocarpus

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Détermination et différenciation du sexe chez l'algue brune *Ectocarpus*

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Chapter 1. General Introduction

I. Origin and maintenance of sex

a. Introduction and definitions

Sex as a means of exchanging genetic material between individuals can be found in all domains of life, from eukaryotes to bacteria and archaea. Nevertheless some fundamental differences exist between eukaryotic sex and bacterial sex. In the latter the transfer of genetic material does not imply the fusion of two gametes (syngamy) and is always unidirectional involving a donor cell and a recipient cell. Any cell can play either of these roles. Another major difference between eukaryote and bacterial sex is the way they generate genetic variability. In bacteria, sex only creates variability through homologous recombination for a small fraction of the genome (Smith *et al*., 1991) whereas in eukaryotes sex produces variability over the entire genome. Contrary to bacterial sex, eukaryotic sex involves an alternation between a haploid and a diploid phase, with meiosis mediating the transition from diploid to haploid phase, and gamete fusion (syngamy) reconstituting a diploid genome. The term "sex" is going to be used in this manuscript to refer to meiotic sex.

Meiotic sex probably evolved only once, during the early evolution of the eukaryotes (Cavalier-Smith, 2002), and is widespread in almost all eukaryotic groups. Meiosis-related genes have been found in all the major eukaryotic supergroups (Malik *et al*., 2008). The ubiquity of sex in eukaryotes strongly suggests that this process has evolutionary advantages, but the origin of sex and its maintenance are still not well understood, and these remain major questions in evolutionary biology. Indeed a number of costs are associated with sexual reproduction making the widespread success of sex and its maintenance in eukaryotes an evolutionary paradox (Maynard Smith, 1978). In the following sections I will describe the hypotheses that have been developed to explain the short- and the long-term advantages of sex that would have allowed sex to evolve in the first place, and then be maintained.

b. The origin of sex

The hypotheses that have been put forward to explain the origin of sex are mostly based on the short-term or direct advantages confered by sexual reproduction and recombination.

DNA repair hypothesis

It has been demonstrated that oxidative stress can trigger sexuality in a broad range of facultatively sexual eukaryotes (Bernstein and Johns, 1989; Davey, 1998; Nedelcu and Michod, 2003; Nedelcu *et al*., 2004) and it is known that oxidative stress can cause physical damage to the DNA (Slupphaug, 2003). It has therefore been proposed that both bacterial recombination and meiotic-sex evolved to repair DNA damage (Bernstein *et al*., 2011). Homologous recombination repair can use the homologous chromosome as a template to repair damage on DNA. Mutation of genes involved in such damage repair systems was shown to not only turn off the ability to repair DNA damages but also to prevent recombination activity during meiosis (Joyce *et al*., 2009; Klovstad *et al*., 2008; Staeva-Vieira *et al*., 2003). In such a system where there is no DNA repair, harmful damage to DNA can accumulate and be transmitted to subsequent generations. Diploidization through syngamy during eukaryotic sex allows homologous chromosomes to be brought together in a single cell, allowing repair of damaged DNA using undamaged copies as a template. This system is particularly effective when there is a double strand damage (Bernstein *et al*., 2011). Nevertheless this hypothesis fails to explain how sex evolved and was maintained in a population containing asexual diploid eukaryotes, where the homologous chromosomes are present allowing homologous recombination repair of DNA damage (Otto and Lenormand, 2002).

Selfish DNA element hypothesis

Another short-term advantage of sex may concern the transmission of selfish DNA elements. Such 'selfish elements' (*i.e*. transposable elements, some genes and all parasitic genetic elements) are known to damage the host (Hurst and Werren, 2001) but provided that they are transmitted rapidly to new hosts before they cause damage to their existing host, sex can improve the transmission of such genetic selfish elements (Hartfield and Keightley, 2012; Otto and Lenormand, 2002). However, this advantage could explain the evolution of

sex but not its maintenance. Indeed, in the long-term, such elements are expected to invade and be found at high frequency in the system. Therefore, in the long-term, the transmission of the selfish elements is predicted to be more efficient in an asexual compared with a sexual population (Hartfield and Keightley, 2012; Otto and Lenormand, 2002). The long-term maintenance of sex cannot therefore be explained simply by transmission of selfish element DNA.

In conclusion, while a number of hypotheses have been put forward, it is still enigmatic why sex evolved in the first place. Possibly, several of these hypotheses may hold true in any given species (*i.e.*, they are not mutually exclusive), and possibly different hypotheses, or combinations of hypotheses, may apply in different species.

c. The maintenance of sex

Despite the widespread occurrence of sex in eukaryotes, sex is costly and explaining its maintenance is an evolutionary paradox known as the paradox of sex (Maynard Smith, 1978). The advantages of sex should counterbalance its costs, otherwise sex would not be so prevalent among eukaryotes. In the following paragraphs the different costs and long-term advantages of sex are reviewed.

Costs of sex

Several factors make sex costly. The first is the well-known two-fold cost of sex. In most sexual eukaryotes, the populations consist of two sexes, with only one (the females) being able to bear offspring. The first cost is therefore the production of males. Indeed, apart from some rare cases, males do not invest significantly in the rearing of offspring, they only contribute to the next generation by providing their genetic information, while females consistently invest in their offspring by providing care and resources (Maynard Smith, 1978). Therefore, for females there is a cost to produce males. The other component of the two-fold cost of sex it is the decrease of genes transmitted to the next generation compared to asexuals. In a sexually reproducing population, offspring inherit half of male and half of female genes. In contrast, asexuals transmit their entire genome to the next generation (Maynard Smith 1971; 1978). The two-fold cost is not absolute, and some conditions can amplify or reduce its effects. The cost can increase if there is more conflict between males for instance in a polyandric system, *i.e.* where one female mates with several males. In this case males compete with each other and can prevent females from mating with other males. On the other

hand, in systems where males invest more resources in their offspring, the cost of sex decreases. In isogamous organisms, for example, the two mating-types are almost undistinguishable and both invest the same resources in offspring by the production of equal-size gametes. Therefore in sexual organisms producing isogametes, the cost of producing males is absent (Lehtonen *et al*., 2012).

Another cost of sex arises from searching for mates. Sexual reproduction needs that both sexes find each other in order to mate, a process that can be highly costly in a low density population. The efficiency in finding a mate was shown to be strongly correlated with the breeding strategy, and in lineages where searches to find a mating partner are ineffective, hermaphroditism has evolved (Eppley and Jesson, 2008). Additionally, the mating process itself involves several risks, such as infection by sexual transmitted diseases, or predator risks associated with having attractive ornaments (Daly, 1978).

There is also a direct metabolic cost of sex. Meiosis is a complicated and time consuming cellular process compared to mitosis. In unicellular organisms meiosis can take up to 100 times longer than mitosis (Otto, 2009). Calculation of the extent of this cost needs, however, to take into account the complexity of the organism because in multicellular organisms the relative time spent performing meiosis compared to life expectancy is not as great as in unicellular organisms.

Finally a more general cost of sex is related to recombination. Even if recombination can create advantageous association of genes more rapidly than it occurs in asexuals (see Box 1), it can also easily break up advantageous combinations. By dissociating positive associations of genes built up by selection, recombination decreases the average fitness of the next generation, which bears the reshuffled genotypes (Maynard Smith, 1978; Otto, 2009; Williams, 1975). This cost of sex is known as the recombination load (Maynard Smith, 1978). Because epistasis (*i.e*. how genes interact and affect a phenotype or the fitness) is a widespread phenomenon, recombination load is probably the most general cost of sex, affecting all organisms that perform recombination (Lehtonen *et al*., 2012).

Long-term advantages of sex

DNA mutations arise due to errors during the replication process or from the physical effects of external stresses. Deleterious mutations are more likely to arise than beneficial mutations. Once a mutation occurs, there is almost no possibility to revert back to the previous un-mutated gene version. Therefore if we consider a finite population of asexuals

with individuals having different level of mutation load, the stochastic loss of the less mutated individuals would induce an accumulation of deleterious mutations over time which is known as the Muller's ratchet phenomenon (Muller, 1964). Because mutation load is detrimental there is a strong selective pressure to eliminate deleterious mutations but the most efficient way to eliminate this load is through sex (*i.e*. recombination). Indeed recombination can mix genomes and therefore re-create individuals with reduced mutation load, avoiding Muller's ratchet. However, in a recent study on the asexual pathogenic *Cryptococcus neoformans*, it was shown that Muller's ratchet can be avoided even in an asexual reproduction system, if processes such as the fusion of identical mating-types, nuclear fusion and meiosis are implemented (Roach and Heitman, 2014).

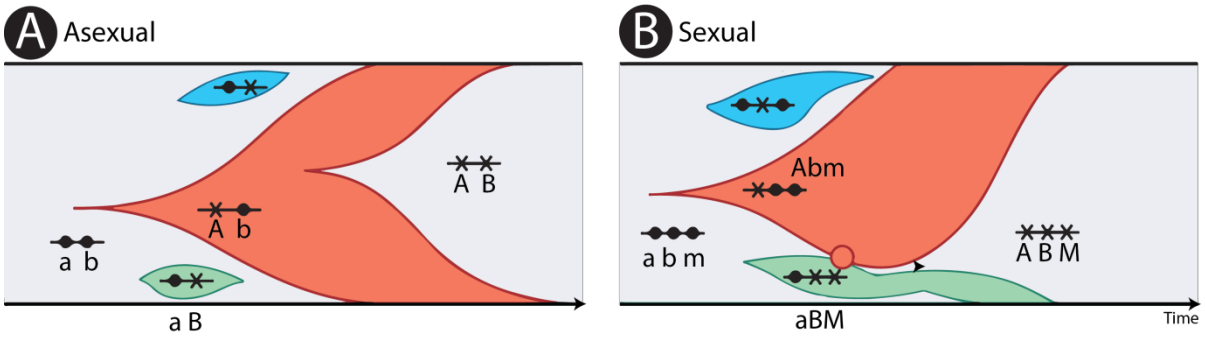
Sex as a generator of variation on which natural selection can act has long been thought to be important for the maintenance of sex (Weismann, 1889). Sex generates different genotypes through recombination and selection then acts on this variation (Burt, 2000). By doing so, recombination and segregation favour the fixation of beneficial mutations and the elimination of detrimental mutations. This idea was developed by Fisher (1930) and Muller (1932) (see Box 1). However, two theoretical studies have suggested that sex does not necessarily imply the generation of genetic variation (Otto, 2009; Otto and Lenormand, 2002) and therefore this hypothesis is unlikely to explain the maintenance of sex in the long-term.

Under certain conditions, such as for example during host-parasite co-evolution, the generation of variable offspring through sex can have an evolutionary advantage. Biotic interactions create a situation for both the host and the parasite in which they are in a continuous arms race. This necessity to continually create diverse offspring to follow an constantly changing environment is known as the Red Queen hypothesis (Bell, 1982; Van Valen, 1973). In a changing environment the fitness of an advantageous genotype can rapidly decrease. For instance in the host-parasite context all genotypes that effectively counter parasites will become more frequent in the next generation. In turn, parasites evolve better arms to infect the hosts making these genotypes less fit. Therefore the generation of genetic variability can allow rapid changes to the biotic environment to be dealt with.

Box 1: Fisher-Muller argument

A. Favorable mutations must be established sequentially in an asexual population. For example, if allele "A" is destined to replace "a", then any favorable alleles that occur at another loci ("B", for instance) can only be fixed if they occur within a genome that carries "A".

B. With sexual reproduction, favorable mutations at different loci can be combined thanks to recombination; this leads to an advantage to modifiers that causes sex and recombination. A favorable allele "B" that occurs with the unfavorable allele "a" can be fixed if it can recombine into association with "A" (red circle); if this recombination requires that a modifier allele "M" be present, then allele "M" will also tend to increase by hitchhiking.



From Barton 2007

d. Asexual reproduction

Although the mechanisms underlying the evolution and maintenance of sex remain somewhat unclear, the ubiquity of this process in eukaryotes clearly demonstrates its advantage over asexuality. Asexual organisms are expected to have low capacity to produce evolutionary novelty and therefore to be an evolutionary dead-end, mainly due to mutation load (Schön *et al*., 2009). The position of asexual lineages on the phylogenetic tree of eukaryotes is consistent with this expectation. Almost all of these lineages occupy terminal branches of the tree (Simon and Delmotte, 2003) with very rare cases of entirely asexual groups. Examples of the latter include bdelloid rotifers, where sex was lost at least 40 million years ago (Birky, 2004). In this group the ancestral duplication of the genome is thought to aid the DNA repair process (Mark Welch *et al*., 2008). Horizontal gene transfer with non-

metazoans and gene conversion may generate genetic variability in these species (Flot *et al*., 2013; Mark Welch *et al*., 2008) and could explain the long-term success of the bdelloid rotifer asexual lineage.

From bacteria to eukaryotes, there are several ways to reproduce asexually, but in this section we will focus on one particular mode of asexual reproduction, known as parthenogenesis. Parthenogenetic reproduction takes place when unfertilized gametes develop into a new individual without the genetic contribution of male. This phenomenon generally concerns female gametes where the size of the gamete provides sufficient resources for independent development. However, exceptions to this rule may occur in some groups, such as the brown algae, where male gametes of certain lineages (such as Ectocarpales) may develop in the absence of fertilization (reviewed in Luthringer *et al*., 2014). Asexual reproduction can allow an organism to avoid some of the cost associated with sex. For instance in asexual organisms recombination load is suppressed and positive genetic associations are stable; asexual reproduction can avoid the cost of finding a mate. In plants studies have determined some direct genetic factors that control asexual reproduction. A variety of genetic systems have been characterised, including both single and multi-locus systems. For instance a unique sex-specific locus controls asexual reproduction in dandelions (van Dijk, 2004) whereas five loci trigger asexuality in *Poa pratensis* (Matzk *et al*., 2005). In both of these examples, asexuality is facultative and is associated with some sexual reproduction.

e. Eukaryotic sexual life-cycles

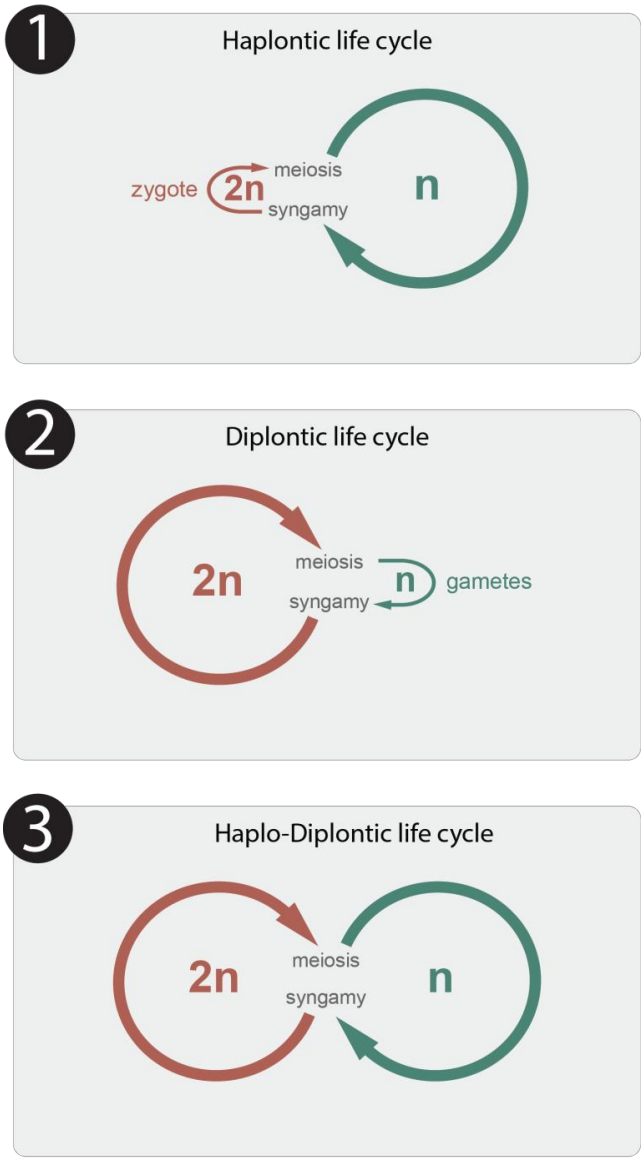
Sexual life cycles in the eukaryotes involve a cyclic alternation between diploid and haploid phases with meiosis mediating the transition from the diploid to the haploid state and cell fusion (syngamy) reconstituting a diploid genome. A wide variety of life cycles can be found in the different groups of multicellular eukaryotes, depending on the time organisms spend in each of the generations and the amount of somatic development. Many types of life cycles exist in nature, but they can be broadly subdivided in three main types: haploid life cycles; diploid life cycles and haploid-diploid life cycles (equal dominance of haploid and diploid phases; Coelho *et al*., 2007).

Haploid life cycles (Figure 1-1): the haploid phase is dominant and the diploid phase is restricted to the short-lived zygote. Meiosis takes place in the zygote, rapidly after syngamy, and produces meiospores. The latter develop into either mating-type plus (+) or

mating-type minus (-) haploid individuals, *i.e*. they are dioicous. Each haploid carries either the + or the – haplotype of the mating-type (MT) locus. These haploid individuals produce haploid gametes that fuse to produce a zygote that is heterozygous for the MT locus. Haploid life cycles can be found in some green algae, fungi and stramenopiles.

Diploid life cycles (Figure 1-2): the diploid stage is dominant and the haploid phase is restricted to the gametic stage. Depending on the species, the diploid stage may have separate sexes (dioecy in plants or gonochorism in animals) but may also be a hermaphrodite, where both sexes are found on the same diploid individual. In the case of separate sexes, the gender is either determined by genetic or by environmental factors (see this Chapter section II). This kind of life cycle is found in almost all Metazoa, but also in diverse eukaryotic groups such as some stramenopiles (*e.g*. the brown algae *Fucus*), alveolates or excavates.

Haploid-diploid life cycles (Figure 1-3): In between haploid and diploid life cycles lies a continuum of haploid-diploid life cycles, where mitosis occurs both during the haploid and the diploid phases. Both stages may remain unicellular (such as in *Saccharomyces cerevisiae*) or may develop into a multicellular organism at both or exclusively at one of the stages (some red, green and brown algae, and some yeasts, respectively). After meiosis, two distinct haploid individuals may be produced (dioicy), which produce either +/female or – /male gametes. Alternatively after meiosis male and female reproductive structures can be found on the same haploid individual (monoicy), as in some brown algae, mosses, liverworts and hornworts. In both cases, fusion of gametes produces a diploid that is heterozygous for the MT/sex locus. In photosynthetic organisms, we usually use the terms gametophyte and sporophyte to refer to the multicellular haploid and diploid phases respectively.



Adapted from Coelho et al. 2007

Figure 1. Main sexual life cycles. Depending on the dominant stage, haploid, diploid or both, sexual cycles can be defined as haploid, diploid or haplo-diploid life cycles respectively. (n=haploid, 2n=diploid).

II. Sex determination in eukaryotes

As described in this Chapter section I.a, meiotic sex has a single evolutionary origin; however, male and female sexes have emerged independently many times in several eukaryotic linages, and by a striking diversity of mechanisms. Sex determination mechanisms are responsible for the triggering and development of the specific male and female sexual

characteristics of an organism. The next sections summarize the different types of sex determination mechanisms found in nature.

a. Epigenetic versus genetic sex determination

The choice between female or male developmental fates can be determined by several different mechanisms. These form a continuum between, at one end, epigenetic sex determination and, at the other extreme, genetic sex determination (GSD) (Gamble and Zarkower, 2012). Note that we are using the definition of epigenetic sex determination according to Beukboom and Perrin (2014) *i.e.*, sex determination and differentiation are epigenetic if sexes/mating types can be produced by the same genotype. This means that both environmental sex determination (ESD) and hermaphroditism are forms of epigenetic sex determination.

Between these extremes there are systems where sex is determined by both genetic and epigenetic factors. In the sea bass, for instance, both temperature and genetic factors influence the level of methylation of the gene that triggers sex determination (Navarro-Martín *et al*., 2011). Epigenetic sex determination includes all systems in which external factors, environmental or social, trigger male or female development. In GSD systems, a region of the genome determines maleness or femaleness. Two different classes of GSD, monogenic and polygenic, can be distinguished based on the number of sex loci involved in the determination of sex. For simplicity, ESD and GSD are going to be treated as two different mechanisms in the next sections, but it is important to bear in mind that this separation is artificial.

b. Environmental sex determination

Environmental factors that trigger sex determination can be divided into two major categories: abiotic and social factors. Photoperiod, pH, oxygen level, food availability and temperature are all environmental cues that can influence the development of one sex or the other. For instance the temperature under which embryos are incubated will determine their sex in most turtles and crocodiles, and in some fishes (Bull and Vogt, 1979; Ospina-Alvarez and Piferrer, 2008; Woodward and Murray, 1993). Also some social cues can determine which sex will develop. In the worm *Bonellia viridis* sex determination depends on where the larva settles. If the larva settles on the seafloor, it develops into a macroscopic female. On the contrary if a larva settles on a female, it migrates inside the female and develops as a microscopic male (Berec *et al*., 2005). In some fishes the presence or absence of the other sex

can trigger the development of a male or female (Godwin *et al*., 2003). In *Crepidula fornicata* individuals create a mound and the development of either males or females depends on their position on this mound (Coe, 1936). Finally social cues can also have an important role in sex determination in plants and mosses (Banks *et al*., 1993; Tanurdzic and Banks, 2004).

Under certain conditions ESD can be advantageous compared with GSD. An organism living in a patchy environment will produce offspring with different fitness in those different areas, for instance females and males do not share the same preferences and each sex will have different fitness in each environment. In this case ESD will increase the fitness of sons and daughters by raising them in their most favourable environment. In such patchy environments, GSD is counter-selected because it can produce females in a male-beneficial environment and conversely for males. However, there is some cost inherent to ESD, such as the fact that ESD can easily generate intersexes and biased sex-ratios (Bull, 1987).

c. Genetic sex determination: polygenic versus monogenic systems

Sex can be determined by genetic factors. In classical monogenic GSD systems such as XY systems in mammals or ZW systems in birds, a single genetic locus is involved in the determination of the sex. In polygenic GSD systems, on the other hand, multiple loci are involved in sex determination. These loci segregate independently and different allelic combinations determine the sex (Bulmer and Bull, 1982; Kosswig, 1964). Polygenic GSD is found in diverse phylogenetic groups, including some fishes, plants, mammals and insects. The genetic mechanisms of sex determination in polygenic GSD vary. For instance in the zebrafish it is, like in all polygenic GSD, the association of several alleles that determines the sex, but in this association of alleles, one of them has a dominant effect for the determination of sex. This same dominant allele can lose its dominance if it is associated with another set of alleles (Anderson *et al*., 2012; Liew *et al*., 2012). Polygenic GSD can also be found in African pygmy mice, where the system involves three sex chromosomes, XYW. The W chromosome evolved from an X chromosome by gaining a female sex-determination allele. In this case only the XY genotype produces males and all other genotypes (XW; XX and WY) produce females (Veyrunes *et al*., 2010). This diversity of mechanisms in polygenic GSD suggests that they evolved independently (Moore and Roberts, 2013). Theoretical work predicts that those polygenic GSD should be transient and quickly evolve towards monogenic GSD systems (Rice, 1986).

Box 2: Sex-chromosome degeneration: population genetics concepts.

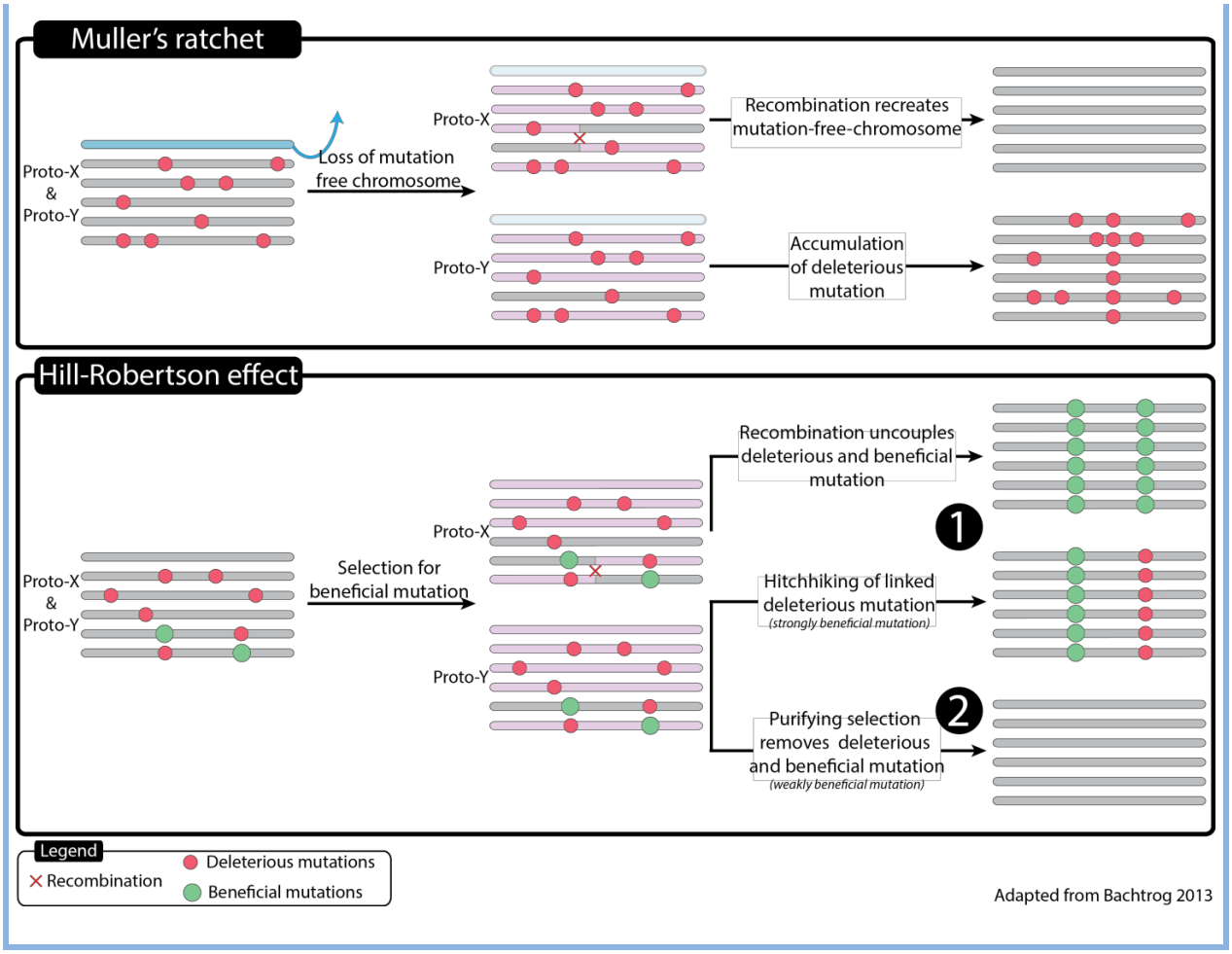
Population genetics concepts applied to sex-chromosome evolution. The schema below represents a population of recombining proto-X and non-recombining proto-Y chromosomes carrying beneficial and deleterious mutations. Genetic degeneration of the non-recombining Y sex chromosome is the result of several processes:

Muller's ratchet:

This process refers to the irreversible accumulation of deleterious mutations in a non-recombining population of chromosomes (Muller, 1964). In a finite population of chromosomes, mutation-free-chromosomes can be lost by chance (genetic drift). Recombination of the proto-X sex chromosomes can then recreate a mutation-free-proto-X-chromosome by associating mutation-free regions from different proto-X chromosomes. This process cannot occur for the non-recombining proto-Y chromosomes, which therefore accumulates deleterious mutations.

Hill-Robertson effect: This process can be subdivided into two distinct processes:

1. If different beneficial mutations are associated with different deleterious mutations, recombination of the proto-X can dissociate negative associations and create positive associations. However, in the absence of recombination the different beneficial mutations compete for fixation which can reduce overall fitness, and deleterious mutation can hitchhike to fixation if they are associated with (genetically linked to) beneficial mutations (a phenomenon also known as "clonal interference"; de Visser, 1999).
2. Here processes is identical to the previous one except for proto-Y chromosomes where beneficial mutation are too weak to counterbalance associated deleterious mutations they will be lost from the population (a phenomenon also known as "ruby in the rubbish"; Peck, 1994)



d. Sex chromosomes and their evolution

In monogenic GSD (M-GSD), a specific region of the genome determines maleness or femaleness. The portion of the genome that determines sex can extend over a large region, corresponding to almost the totality of the chromosome, such as with the human sex chromosomes (Goodfellow *et al*., 1985), or can be a single polymorphism, as in the tiger pufferfish (Kamiya *et al*., 2012).

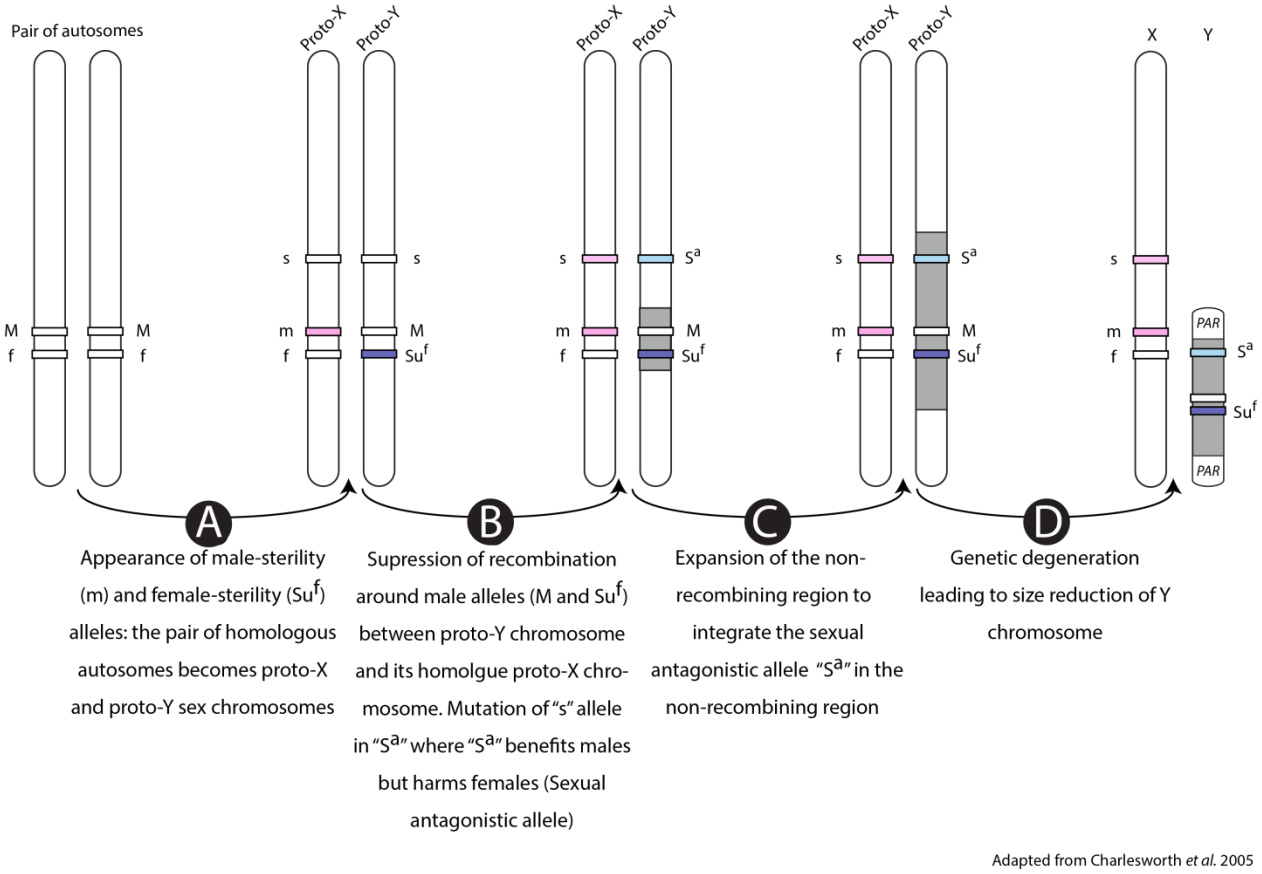
Sex chromosome evolution has been the object of research for many years. The currently accepted theory for the evolution of sex-chromosomes predicts that sex chromosomes evolved from a pair of autosomes, the process beginning with the acquisition of a sex-determining locus, for example a male-determining gene. Then a recessive male-sterility mutation appeared on the proto-X chromosome and a dominant female-sterility mutation appeared on the proto-Y chromosome, which induced the emergence of separate sexes in a hermaphrodite population (Figure 2-A). In this situation, as each of the proto-sex

chromosomes carries a sterility mutation, both mutations could be located on the same chromosome if a recombination event takes place between the two loci, creating sterile individuals. Therefore there is strong selection pressure to suppress recombination between these two regions on the proto-Y and the proto-X (Figure 2-C). Once recombination is lost, the efficiency of natural selection to incorporate beneficial mutations and to purge deleterious mutations from the non-recombining region is decreased. Indeed in non-recombining chromosomes genetic linkage between beneficial and deleterious mutations probably leads to the accumulation of deleterious mutations (see Box 2, Muller ratchet and hitchhiking effects) and reduced purifying selection (see Box 2, Hill-Robertson effect). A decrease in the strength of selection can also occur due to the reduction of the effective population of sex chromosomes: the proto-Y chromosome is only inherited by males (the effective population is half that of an autosome) and the proto-X is preferentially inherited by females (the effective population size is three quarters that of an autosome). As a result, non-recombining sex chromosomes are more permissive for the accumulation of transposable elements, inversions and mutations (Bachtrog, 2013 and Box 2). In the short-term those modifications can cause expansion of the non-recombining sex-determining region by the accumulation of mutations and transposable elements, and can create a proto-Y that is bigger than the proto-X.

The non-recombining region can expand as a result of sexually antagonistic selection (Figure 2-C), which occurs when alleles have different fitness in males and females. If a sexually antagonistic gene occurs near the non-recombining sex-determining region (SDR), one way to resolve conflict is to fix the advantageous gene in the good sex and remove it from the disadvantaged sex. Such fixation of a sexual antagonistic (SA) gene into one sex can be achieved by the expansion of the non-recombining region (the SDR) to include this gene (Rice, 1996). Theoretical models predict that SA alleles should accumulate close to the non-recombining sex-determining region (Charlesworth *et al*., 2014; Otto *et al*., 2011) and drive the evolution and expansion of the this region. Non-recombining regions, however, often do not extend over the entire length of the sex chromosome; part of the sex chromosome, named the pseudoautosomal region (PAR), continues to recombine and this maintains homologous regions between the heteromorphic sex chromosomes. The cessation of recombination in sex chromosomes often occurs in a stepwise fashion, creating so-called evolutionary strata. These evolutionary strata have different levels of divergence according to the time spent without recombining (Bergero and Charlesworth, 2009). Strata have been described in a number of organisms regardless of the type of sexual system, including animals (Handley *et al*., 2004;

Lahn, 1999; Vicoso *et al*., 2013a), plants (Bergero *et al*., 2007; Wang *et al*., 2012) and fungi (Votintseva and Filatov, 2009)

In systems where sex is expressed in the diploid stage (XY and ZW systems) because of the accumulation of deleterious mutations and the decrease of adaptation, sex-specific non-recombining chromosomes (*i.e*. the Y, found only in males, and the W, found only in females; Figure 2-D) experience a genetic degeneration over the long term (see above and Box 2). In contrast the absence of degeneration on X and Z chromosomes is easily explained by the maintenance of recombination in the sex carrying the homomorphic sex chromosomes (XX females and ZZ males), which allows efficient elimination of deleterious mutations. Loss of genes and reduced gene expression in the degenerating sex chromosomes (Y and W) creates disequilibrium between female and male for the non-degenerated sex chromosomes (X and Z). For instance in XY system, females carry two X chromosomes and males only one, leading to a bias in X-linked gene content between sexes. This bias may be compensated for by adjusting the expression of the X chromosome genes in females and males, a process called dosage compensation (Straub and Becker, 2007).



<u>Figure 2</u>. Sex chromosomes evolution in an XY system. In a hermaphrodite population, a pair of homologous chromosomes carries the "M" and "f" alleles. (A) "M" mutates into a recessive male-sterility allele (m) which

causes the emergence of females and the dichotomy of proto-X and proto-Y chromosomes. On the proto-Y, "f" mutates into the dominant "Su^{f}" allele, causing female-sterility and the appearance of males. (B) Between the proto-Y chromosome and its homologue the proto-X chromosome, suppression of recombination around male alleles (M and Su^{f}) is favored creating a non-recombining sex determining region (grey region). The "s" gene undergoes mutation on the proto-Y chromosome to create a sexual antagonistic allele (S^{a}) that benefits the male but harms the female. (C) On the male proto-Y chromosome expansion of the non-recombining region to include S^{a} is favored. (D) The lack of recombination on the Y chromosome induces accumulation of transposable elements, genetic degeneration and gene loss resulting in a smaller male Y chromosome. The non-recombining region is not spread throughout the Y chromosome, the pseudoautosomal region (PAR) can still recombine with the corresponding region on the X. Adapted from Charlesworth *et al*., 2005.

Heteromorphic sex chromosomes were the first and the most studied sex chromosomes, probably because they were the easier to identify in karyotypes. However, it is important to note that loss of recombination in sex chromosomes does not always create heteromorphic sex chromosomes. For example, in ratite birds, and Boridae snakes sex chromosomes remain undifferentiated despite being old (Vicoso *et al*., 2013a, 2013b). There are several hypothetical explanations for the absence of obvious genetic degeneration in such systems. Low levels of sexual dimorphism and sexual selection could limit the expansion of non-recombining regions, avoiding the deleterious effect of losing recombination (Rice, 1984). Other hypothesis that have been proposed include occasional X-Y recombination, which can eliminate accumulated deleterious alleles (Stöck *et al*., 2011), and resolution of sexual antagonism not by incorporating the SA allele in the SDR but through differential expression of PAR genes between sexes leading to sex-biased expression (Vicoso *et al*., 2013b).

Sexual reproduction is widespread in eukaryotes but loss of sex chromosome recombination has evolved independently and repeatedly across the different eukaryotic groups. Comparative studies between different systems have shown that recombination suppression is a common feature in sex chromosome evolution and therefore a clear example of evolutionary convergence. These independent evolutionary events of loss of recombination have led to an extraordinary diversity of sex chromosomes which will be described in the next section

e. Types of sex chromosome system

Eukaryotic monogenic GSD systems include three types of chromosomal sex determination system: the diploid XY and ZW sex-determination systems, and the haploid UV sex-determination systems. The XY system, which is found in mammals, is defined as a male heterogametic system, where males carry the two different sex chromosomes and females the two homologous X chromosomes. Conversely in ZW systems, defined as female heterogametic, it is the female that carries the two different sex chromosomes and males the two homologous Z chromosomes. Finally UV sex chromosomes can be found in some algae and bryophytes, and in such systems sex is expressed at the haploid stage (Figure 3, Bachtrog *et al*., 2011). In UV systems, females carry a U chromosome, whereas males carry a V chromosome. These three systems share many common characteristics, but there are important differences between them that have major evolutionary and genomic consequences. In diploid sex chromosome systems, the inheritance of sex chromosomes between sexes (Y and W are sex-specific and X and Z are preferentially found in females and males respectively) results in a specific pattern of distribution of SA genes in those chromosomes. For instance in the XY system, males are always in a heterozygous state for sex chromosomes, which allows recessive male-beneficial alleles to accumulate on the X chromosome because they are always expressed in males. Because the X chromosome is always in the homozygous state in females, male-beneficial alleles that accumulate on the X chromosome must have low costs for females. Also, dominant female-beneficial alleles can accumulate on the X chromosome because this chromosome is preferentially found in females (Charlesworth *et al*., 1987; Rice, 1984). A corresponding, but inverse, process is expected in ZW systems. X and Z chromosomes recombine and therefore avoid genetic degeneration, but in UV systems both sex chromosomes have lost their capacity to recombine. In UV systems the sex of the haploid meiotic offspring is determined by whether it carries a female (U) or male (V) chromosome. Importantly, there is no homogametic sex, and both the U and V are always hemizygous in the diploid phase (UV). The relatively important time spent in the haploid phase of the life cycle is expected to expose the U and V sex chromosomes to purifying selection presumably limiting the degeneration of such sex chromosomes (Bull, 1978), but also confers unique genetic and evolutionary features compared to diploid systems. As a result of haploid purifying selection, the non-recombining regions of UV sex chromosomes are expected to evolve through the addition of genetic material by duplication or translocation instead of genetic degeneration (Bull, 1978). Until recently very little

sequence data has been available for UV systems: only one sex chromosome in the liverwort *Marchantia* (the V) (Yamato *et al*., 2007); a UV pair of unknown age in the green alga *Volvox* (Ferris *et al*., 2010), some fragmentary data for the moss *Ceratodon* (McDaniel *et al*., 2013) and, more recently, the UV sex chromosomes of the brown alga *Ectocarpus sp*. (Ahmed *et al*., 2014; see Chapter 2).

The mechanisms and processes that lead to the appropriate and unambiguous development of separate sexes and reproductive structures are known as sex determination. The process of sex determination is complex and dynamic, with a diversity of cues (social, genetic, environmental) that trigger cascades of interacting factors. The regulation of the factors involved in sex-determination has to be precise in order to produce individuals with unambiguous sex and reproductive structures. For example, in organisms with diploid sex determination systems, the heterogametic sex possesses all the genetic information necessary to produce both sexes.

In monogenic GSD the triggering genetic factor is contained in the non-recombining region of the sex chromosomes, and is surprisingly poorly conserved between lineages. The downstream sex-determining elements, in contrast, are more conserved across lineages (Graham *et al*., 2003). The factors downstream in the sex-determining cascade can be conserved even between ESD and GSD, as it was shown by comparing the *doublesex* gene of the crustacean *Daphnia magna* with that of several insects (Kato *et al*., 2011). *Doublesex* is part of the DM-domain gene family, and is highly conserved downstream of the male-determining factor in all animals, from vertebrates to cnidarians (Miller *et al*., 2003; Raymond *et al*., 1999). Of the several types of sex-determining factors, high mobility group (HMG) proteins are probably the most studied. HMG proteins, such as the SRY protein, are transcription factors that carry an HMG-box domain and these proteins trigger the sex determination pathway in almost all mammals (Kashimada and Koopman, 2010). Interestingly a HMG protein has also been found to be involved in sex determination in fungi (Idnurm *et al*., 2008) and a member of this family was identified in the female MT locus in *Volvox* (Ferris *et al*., 2010).

The downstream cascade of effectors involved in the complex pathways of male and female sex-determination ultimately establish the phenotypical characters that allowed the two sexes to be distinguished physiologically and/or morphologically. The next section describes why two genders evolved in the first place and then how differential selective pressures between genders can lead to the evolution of sexual dimorphism.

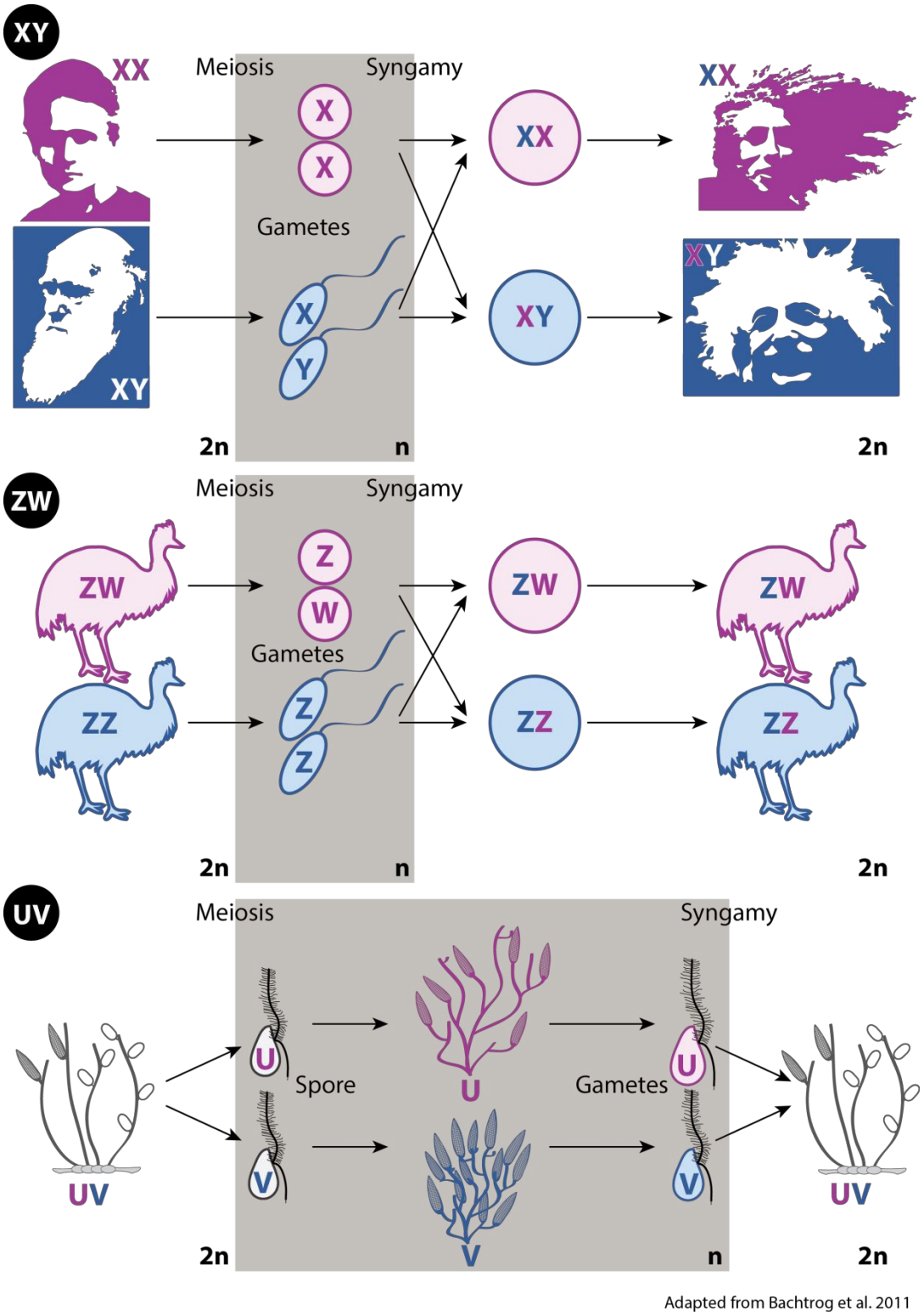


Figure 3. The three types of chromosomal sex determination found in eukaryotes. XY system where the Y is specific to males; **ZW** system where the W is confined to females. In both systems sex is expressed in diploid individuals. On the contrary, in **UV** systems sex is expressed in the haploid phase where U chromosome is limited to females and V chromosome to males (Bachtrog *et al*., 2011).

III. From sex determination to sexual differentiation: the evolution of sexual dimorphisms

a. Evolution of mating types

Sex is clearly an advantageous mechanism but what are the uses of mating-types and sexes? Indeed why is there restriction in terms of sexual partner? Why not mix genomes with all possible partners? Instead sexual reproduction restricts mating between compatible mating-types or sexes, often + and – or female and male. This section will focus on the evolution of dichotomic mating-types and sexes (+ and -; female and male), and will rapidly describe the different hypotheses aimed at explaining the origin of two gamete classes (for a complete review see Billiard *et al*., 2011).

The "by-product" model suggests that mating types evolved as a by-product of the molecular mechanisms for gamete recognition. In this model we have to assume that an initial population produced undifferentiated gametes, all of them both producing pheromone and carrying pheromone receptors, so that any gamete could fuse with any other gamete. In this population the model explains the evolution of separated sexes by the differential loss of either pheromone production or the pheromone receptor. Such a differential loss, leading to two classes of gametes (pheromone producers and pheromone sensitive) is assumed to increase the chance of gametes fusing, compared to undifferentiated gametes. In this model undifferentiated gametes are expected to experience self-saturation of the pheromone receptor by self-production of pheromones, which can prevent gamete fusion (Hoekstra, 1982). However, in some fungal species, gametes can produce both pheromones and receptors (Billiard *et al*., 2011).

The "selfish element" model proposes that selfish genetic elements can promote the fusion of cells. If a population of cells includes selfish-element-infected and uninfected individuals, selfish elements can have an evolutionary advantage in promoting the fusion of those cells in order to invade the entire population. In such a population, a system of recognition between infected and uninfected cells needs to evolve, allowing the selfish-element to spread efficiently. In the "selfish element" model sexual reproduction and syngamy between two classes of gametes evolved together (Bell, 1993; Hoekstra, 1990 and see this Chapter section I.b).

The "inbreeding avoidance" model proposed that the two classes of gametes evolved in order to avoid the costs of mutation load due to mating between genetically related

individuals (Charlesworth and Charlesworth, 1979; Uyenoyama, 1988a, 1988b). The main problem with this model is that it applies only to diploid life cycles. Haploid or haploid-diploid cycles have a significant haploid stage and hence should not suffer from inbreeding depression.

The "organelle inheritance" model hypothesized that the cost of intra-genomic conflict should favour the evolution of two mating-types. The cost of mixing organelles from several individuals could have been avoided by evolving uniparental inheritance of organelles. Then, a system should evolve to allow organelles carriers and non-carriers to recognize each other (Hurst and Hamilton, 1992; Hutson and Law, 1993; Yamauchi, 2003). Despite large support across several taxa, where sexes and mating-types correlate with the inheritance pattern of organelles, a lot of counterexamples exist. In the brown alga *Ectocarpus,* even if mitochondria are only inherited from females gametes, chloroplasts in zygotes are inherited from both parents, with a mosaic distribution of parental chloroplasts (Peters *et al*., 2004a). Other examples contradict this hypothesis, such as random uniparental inheritance of organelles (slime moulds, Silliker *et al*., 2002), or equal inheritance of organelles followed by random suppression to mediate subsequent uniparental inheritance (Pseudo-nitzschia, Levialdi Ghiron *et al*., 2008).

Under the "developmental switch" model, mating-types evolve because each mating-type locus controls the expression of sex-specific transcription factors that are complementary and function to trigger sporophytic development (Perrin, 2012). In agreement with this model, each mating-type of *Chlamydomonas* produces a transcription factor and the two transcription factors heterodimerize to trigger the sporophytic program (Lee *et al*., 2008). However in some brown algae, unfused haploid gametes can germinate to produce haploid sporophytes (reviewed in Luthringer *et al*., 2014; Annexe 1), and in this case the identity of the generation is not dependent on its ploidy level.

Eukaryotic mating-types have probably evolved independently and repeatedly and their evolution predated that of differences in gamete size (anisogamy) (Hoekstra, 1987; Togashi and Cox, 2011). Anisogamy induces the first sexual conflict for parental investment, which is the basis for the evolution of sexual dimorphism. In the next section the different hypothesis for the evolution of anisogamy are discussed.

b. Evolution of anisogamy

The evolution of anisogamy established the fundamental basis for maleness and femaleness and led to an asymmetry in resource allocation to mating and offspring, leading in many cases to sexual selection. The evolution of the differences in gamete size is probably at the origin of other differences between sexes. According to their size, different types of gametes can be found ranging from identical gametes (isogamy), to differentiated gametes with production of large and small gametes (anisogamy) that can, in some cases, loose motility of the largest gamete (oogamy).

Anisogamy and oogamy have arisen repeatedly across the eukaryotes and these systems are thought to have derived from simpler isogamous mating systems in ancestral unicellular species, when a mutant with a gamete size different from normal invaded an isogamous population (Kirk, 2006; Parker *et al*., 1972). Extensive theoretical work has proposed that the mechanism for the evolution of anisogamy involved linkage of the gamete size gene to the mating type locus (Charlesworth, 1978). Empirical data to validate these predictions is still scarce, and the molecular basis of gamete size control and its link to sex determination remains unclear (Hiraide *et al*., 2013)

Similarly, the underlying reasons why females produce large gametes and males small gametes remain an important question in evolutionary biology and there are currently three major hypotheses to explain the evolution of anisogamy.

The first proposes that anisogamy evolved from intracellular conflicts (Murlas Cosmides and Tooby, 1981; Togashi and Cox, 2011). This theory is based on the idea that genes are the unit of selection. In this context, the inheritance of nuclear and cytoplasmic genetic material can be under conflict. Indeed cytoplasmic elements (mitochondria, chloroplast and intracellular parasites) can be genetically different and therefore selection can favour the spread of certain cytotypes to the detriment of others. This conflict between cytotypes can lower the overall fitness of the cell and therefore this can amplify the conflict between cytoplasmic genes and nuclear genes. The conflict between cytotypes may select for organelles that are more numerous in zygotes, and if this number of organelles in zygotes is directly correlated with the number in gametes and consequently with gamete size, selection for cytoplasmic genes would favour an increase in gamete size. Once the increase in gamete size is sufficient to carry the cytoplasmic resources necessary for early zygotic development, selection would act to enhance the transmission of nuclear genes. The selection for tiny gametes that are inexpensive to produce would allow to increase the quantity of gametes and

therefore to enhance the inheritance of nuclear genes (Lessells *et al*., 2009; Murlas Cosmides and Tooby, 1981). However, intracellular conflict alone is unlikely to explain the evolution of anisogamy but this process may have played a role in maintaining anisogamy (Lessells *et al*., 2009; Parker, 2014).

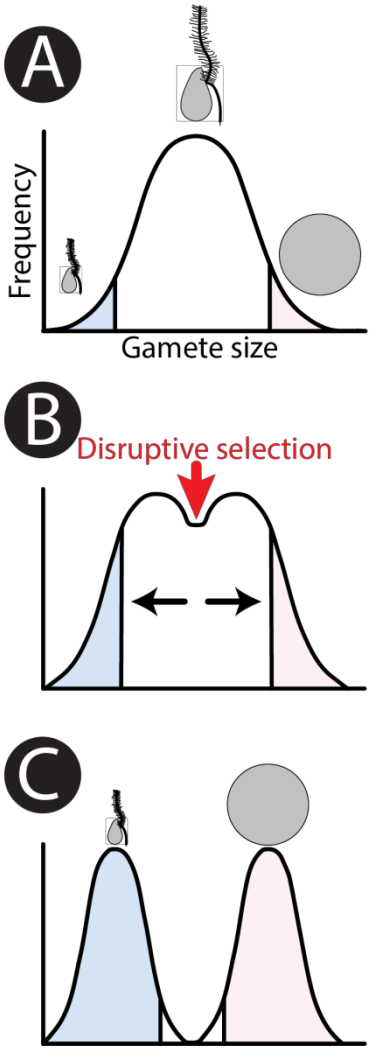
The second hypothesis, proposed by Parker, Baker and Smith (1972) and known as the "gamete competition theory", will be referred to as the PBS model. In the PBS model, the main factor influencing the evolution of anisogamy is the strength of the relationship between zygote fitness and zygote size. In their model, three strengths of this relation were tested: weak, intermediate and strong. If there is a weak relationship between zygote size and zygote fitness, there would be directional selection for the production of only small gametes. In this case, the fitness advantage of producing large gametes does not counterbalance the benefit of producing small gametes. On the other hand, when the relationship is strong there is directional selection for the production of large gametes. Finally, it is the intermediate relation that allows the evolution of anisogamy with disruptive selection (Lehtonen and Kokko, 2011; Lessells *et al*., 2009; Parker *et al*., 1972) (Box 3).

The third hypothesis, known as the "gamete limitation theory", proposes that anisogamy evolved in response to selection forces that acted to increase the rate of gamete fusion. In a broadcast spawning species, the rate of encounter between gametes is critical and a lot of gametes remain unfertilized. To counteract this gamete limitation and fusion limitation, motility or the size of eggs can be increased in order to improve the chance of encounter (Lessells *et al*., 2009; Levitan, 1996).

The "gamete competition" and "gamete limitation" theories are probably the most convincing, and recent studies and models have tried to unify those theories for the evolution of anisogamy (Lehtonen and Kokko, 2011). The evolution of anisogamy provided the first occasion for sexual conflict to arise. Indeed males, by producing small gametes, "parasitise" the parental care of the females, which is ensured by the production of large gametes. This initiation of sexual conflict between sexes by the evolution of anisogamy should therefore be at the basis of the evolution of sexual dimorphism (Lehtonen and Kokko, 2011; Parker, 2014).

Box 3 : Evolution of anisogamy

Disruptive selection of gamete size where intermediate gametes are selected against.



A. An original distribution of gamete size, with the majority of gametes having an intermediate size. Based on the Parker model intermediate gametes are too large to be produced in great quantity but too small to ensure early embryogenesis. At the extremities of the distribution, tiny and big gametes are underrepresented. Tiny gametes are inexpensive to produce and therefore can be produced in great quantity. On the contrary big gametes are costly to produce but carry sufficient resources to support the zygote through early embryogenesis.

B. Selective pressure counter-selects intermediate gametes and favors both small and large gametes at the extremes of the distribution. This selection for extreme gametes size is called disruptive selection.

C. After the action of the disruptive selection, the gamete population is only composed of two kinds of gametes: tiny (spermatozoids) and large (ova).

c. From anisogamy to morphologically different males and females: evolution of sexual dimorphisms

It is largely admitted that the initiation of sexual conflict, sexual selection and "attribution" of sex roles evolved from a cascade of evolutionary events initially arising from anisogamy (Parker, 2014; Schärer *et al*., 2012; but see Ah-King, 2012). In anisogamous organisms the gamete production strategy is not the same in males and females, with the male producing many more gametes than females. This difference leads to a sexual conflict known

as the Bateman principle, where males can increase their reproductive fitness by increasing the number of gametes produced and the number of matings, a strategy that females cannot adopt (Bateman, 1948). The best way for females to increase their fitness is to select the best mate to produce fitter offspring. The eagerness of males and the choiceness of females, together with investment in parental care, are the parameters that define the sex roles. The eagerness of males leads to competition between males for access to females, which is one of the components of sexual selection. Another component of sexual selection is the females' choice of mate.

Sexual selection was first proposed by Darwin in 1871 to explain how sexual dimorphic traits evolved. As explained above sexual selection acts differently in males and females. In the former, male-male competition for access to females creates sexual selection for the evolution of sexual characters related to attractiveness or the ability to increase mating success or reduce the mating success of other males. The evolution of traits improving the attractiveness of males evolved because of female choosiness (O'Donald, 1980). Female choices are made based on traits that indicate the biological fitness of males. Such indicators are cues for "good genes" that can enhance the fitness of offspring, giving the opportunity for females to invest in the production of "good" offspring. Those "good genes" found in males can directly benefit females by improving parental care or by providing females with good territories (*e.g.*,Williams, 1966; Orians, 1969), but they can also benefit the offspring by providing them with good genes (Grafen, 1990; Hamilton and Zuk, 1982; Zahavi, 1975). This "good gene" benefit is known as the "runaway" process when it involves sexually selected characters because females produce sons that are themselves enhanced in their attractiveness. Such processes lead to the evolution of male ornaments, such as the tail of male peacock.

IV. Using the brown algae to study the evolution of the sexes

Brown algae are photosynthetic organisms found almost exclusively in marine environments, with the majority of species diversity being found in cold water regions. Brown algae are mainly found in the intertidal zones, which is a particularly stressful environment (important abiotic variations), leading to many interesting adaptations. Brown algae are one of the rare groups where complex multicellularity has evolved, and produce an astonishing diversity of morphologies, ranging from microscopic organisms to seaweeds that may attain 50 meters long. These large brown macroalgae, also known as kelps, are of important

ecological importance because they create sub-marine forests that shelter an important diversity of organisms. In addition to their ecological interest, brown algae have an important economic interest, with a wide range of uses from food to research for active molecules (McHugh, 2003).

The evolutionary position of brown algae has also stimulated research on this group. Brown algae belong to the Stramenopiles, a group which is phylogenetically almost as distant from the green lineage (Archaeplastidia) as it is from animals (Opishokonts) (more than 1 billion years; Figure 4). This distant phylogenetic position is particularly interesting to assess the universality or novelty of some of the processes driving the evolution of sex determination.

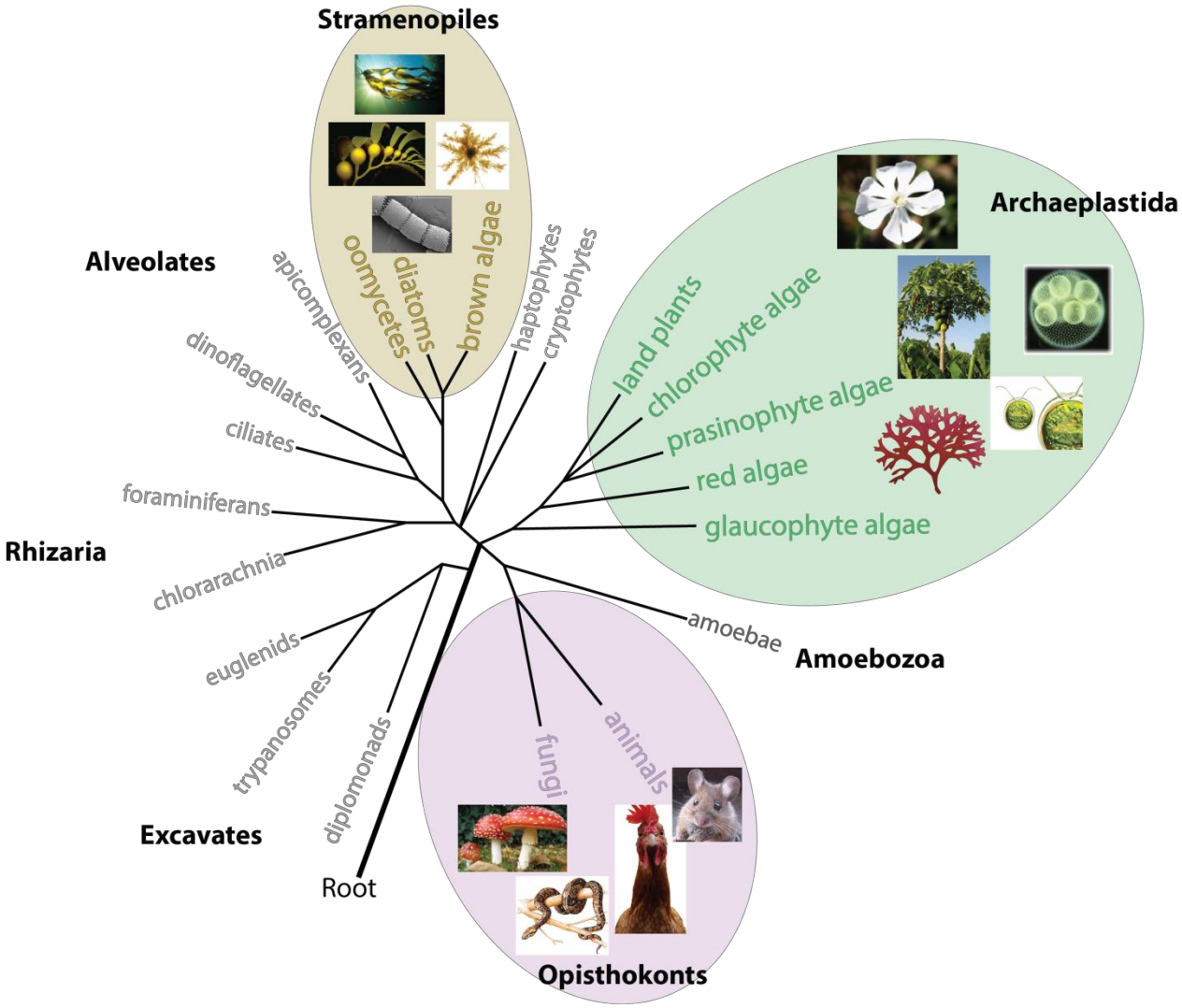


Figure 4. Eukaryotic tree. (adapted from He *et al*. 2014). Brown algae belong to the stramenopile phylum, phylogenetically distant from opisthokont (animals and fungi) and archeplastida phyla (green lineage & red algae).

a. Brown algae display a diversity of types of sexual system

An additional advantage of the brown algae, in the context of the evolution of sex determination, is the fact that they exhibit both an extraordinary diversity of types of life cycle and a wide range of different sexual systems (Luthringer *et al*., 2014; Silberfeld *et al*., 2010) For example, sexuality is expressed during the diploid phase of the life cycle in brown algae with diploid life cycles (dioecy) such as the fucoids, whereas it is the haploid gametophyte generation that exhibits sexuality (dioicy) in algae such as *Ectocarpus,* that have haploid-diploid life cycles (Luthringer *et al*., 2014). The selective pressures leading to the evolution of these different systems are distinct: whilst dioecy might evolve from monoecy to limit inbreeding (due, in the latter, to the fertilisation of female gametes by male gametes produced

by the same organism), this is unlikely to be the case for dioicy because deleterious mutations should be efficiently purged during the extensive haploid phase of the life cycle. Similarly, genetic sex determination is expected to operate differently, with XY or ZW systems occurring in dioecious species but UV systems occurring in dioicous species. When the different types of brown algal life cycle are mapped onto a phylogenetic tree, the distribution pattern suggests that there has been considerable switching between different life cycle strategies and sex chromosome systems during the evolution of this group (reviewed in Cock *et al*., 2014). Phylogenetic analysis indicates that dioicy was the ancestral state in the brown algae, and the transition to dioecy presumably required an intermediate state of co-sexuality (*e.g*. monoecy) with epigenetic sex differentiation (as opposed to genetic sex determination).

b. Brown algae exhibit a broad diversity of levels of sexual dimorphism

Several sexually dimorphic traits have been described in brown algae (Luthringer *et al*. 2014). These can be divided into two main classes: 1) differences between male and female gametes and 2) differences between the male and female gamete-producing stage of the life cycle (the gametophyte generation in species with haploid-diploid life cycles).

Brown algae exhibit, within a monophyletic group, a broad range of levels of gamete sexual dimorphism, ranging from isogamy (*e.g*. *Scytosiphon lomentaria*) to oogamy (*e.g*. *Fucus*) (Annexe1: Luthringer *et al*., 2014). The phylogenetic distribution of gamete size dimorphism has led to the surprising hypothesis that oogamy was the ancestral state in brown algae (Silberfeld *et al*., 2010). If this hypothesis is correct, it suggests that it may be possible for oogamy to evolve towards isogamy, despite the fact that this type of transition is difficult to explain from a theoretical point of view (see in this Chapter section III.b and Togashi *et al*., 2012). Interestingly, gamete size differences in anisogamous and oogamous brown algal species are likely to determine whether a gamete is capable of parthenogenesis. Usually both male and female gametes of isogamous brown algal species are capable of parthenogenesis, whereas only the female gametes of anisogamous species are parthenogenetic (*i.e*. in the latter parthenogenesis is a sexually dimorphic trait; see Clayton and Wiencke, 1990; Ramirez *et al*., 1986 for exceptions). In oogamous species, the large female gamete is specialised for zygote production and is no longer capable of initiating parthenogenetic development.

Female and male gametophytes can also exhibit sexual dimorphisms. In the orders Laminariales, Desmarestiales, Sporochnales, and Tilopteridales microscopic gametophytes

exhibit significant sexual dimorphisms, with females being composed of large cells and males of small cells. This dimorphism allows the morphological identification of females and males in these orders (Müller *et al*., 1985; Sauvageon, 1915; Schreiber, 1932). Sexes can also exhibit differences in terms of the timing of sexual maturation. In male gametophytes of the kelp *Alaria crassifolia* antheridia ripen after 4 days under favourable conditions, whereas females require 10 days (Nakahara and Nakamura, 1973). In some cases gametophytes exhibit their sexual dimorphisms under specific, usually extreme, environmental conditions. For instance temperature can differentially influence the survival of male and female individuals of some species (Cosson, 1978; Funano, 1983; Lee and Brinkhuis, 1988; Nelson, 2005; Norton, 1977; Oppliger *et al*., 2011). Salinity is another abiotic factor that may influence the sex ratio of some brown algae (Norton and South, 1969; Valeria Oppliger *et al*., 2011).

In brown algae the level of sexual dimorphism is relatively low in comparison with animals, a situation similar to that found in land plants. On the latter it was hypothesized that the low level of sexual dimorphisms is due to the recent evolution of dioecy, and therefore the lack of sufficient time for sexual selection to establish extensive sexual dimorphisms (Barrett and Hough, 2013). However, in brown algae dioicy probably evolved much earlier (Figure 1 in annexe 1), and therefore the latter hypothesis is unlikely to explain the apparent low level of sexual dimorphism in brown algae. Nevertheless, the reproductive biology of brown algae can account for the absence of ostentatious sexual dimorphisms. Indeed, in animals sexes have direct contact with each other allowing sexual selection to strongly affect male and female behaviour and shape sexual dimorphisms (see this Chapter section III.c for more details). On the contrary, in brown algae sexes release their gametes into the surrounding medium (broadcast spawning) and there is only indirect contact between sexes, which provides less opportunity for sexual selection to occur. Consistent with this idea, it was shown that in broadcast spawning organisms, the level of sexual dimorphism is lower than in organisms that have direct contact between sexes during copulation (Levitan, 1998; Strathmann, 1990).

The growing interest in brown algal research has resulted in the development of a model for the study of these organisms. In 2004, Peters and colleagues (Peters *et al*., 2004b) proposed *Ectocarpus* as a model organism for brown algal studies. *Ectocarpus* is small filamentous marine brown alga, presenting several advantages: easy to culture in laboratory (short life cycle; small size); facility to carry out genetics analysis (crosses; genetic tools available) and a relatively small, sequenced genome (Cock *et al*., 2010). A number of tools

have therefore been developed for this organism, including quantitative PCR (Le Bail *et al*., 2008), classical genetics (Peters *et al*., 2008), proteomic techniques (Ritter 2010), a genetic map (Heesch *et al*., 2010), RNA-seq approaches (Lipinska *et al*., 2013) and additional techniques under development such as transformation, RNAi and a TILLING mutant collection. Furthermore, *Ectocarpus* has the advantage of having a haploid-diploid life cycle, where both sporophyte and gametophyte are multicellular. This feature allows the study of molecular mechanisms underlying the alternation between the gametophyte and the sporophyte generations (Coelho *et al*., 2011; Peters *et al*., 2008) but also provides an unique opportunity to study a UV sex chromosome system (see Chapter 2). The *Ectocarpus* life cycle involves alternation between two independent multicellular heteromorphic generations: the gametophyte (GA) and the sporophyte (SP) (Figure 5). The sporophyte generation consists of prostrate filaments composed of round and elongated cells and of upright filaments, while gametophytes have highly branched upright filaments composed of cylindrical cells. The dioicous gametophytes, when mature, produce either male or female gametes within plurilocular gametangia. The gametes, after release into the surrounding medium, fuse with a gamete of the opposite sex (Figure 5-A) to give rise to the diploid sporophyte. The latter generation produces plurilocular and unilocular sporangia, containing mito-spores (produced by mitosis) and meio-spores (produced by meiosis), respectively. A single meiotic event takes place inside each unilocular sporangium, producing 4 daughter cells that, after several mitoses, produce 50 to 100 meio-spores (Figure 5-B). After release, these meio-spores germinate into new gametophytes, completing the sexual life cycle of *Ectocarpus*. The mito-spores released from plurilocular sporangia develop into a new, clonal diploid sporophyte (Figure 5-C). Another mode of asexual reproduction involves the production of partheno-sporophytes (pSP). When gametes are not able to find a partner to fuse with, they can develop parthenogenetically into fully functional partheno-sporophytes (Figure 5-D). Partheno-sporophytes are morphologically (and functionally, Peters *et al*. 2008) indistinguishable from diploid sporophytes, and produce unilocular sporangia and plurilocular sporangia. As with the reproductive organs of the diploid sporophyte, spores from unilocular sporangia develop into gametophytes (Figure 5-E) and those from plurilocular sporangia germinate into new partheno-sporophytes (Figure 5-F) (Müller, 1967; Bothwell *et al*. 2010).

In the gametic sexual dimorphism "gradient" displayed by the brown algae, *Ectocarpus* is in a particularly interesting position. Indeed, while the size of male and female gametes has been considered to be the same (but see Chapter 4), the behaviour and

physiology of *Ectocarpus* gametes are strongly dissimilar (Berthold, 1881; Müller, 1972). Shortly after release, female gametes settle and produce pheromones to attract male gametes, which swim for longer and are attracted by the pheromone. Hence *Ectocarpus*, and other closely related brown algae such as *Scytosiphon*, are in a key position for the study of the molecular mechanisms associated with early events in the evolution of sexual dimorphism.

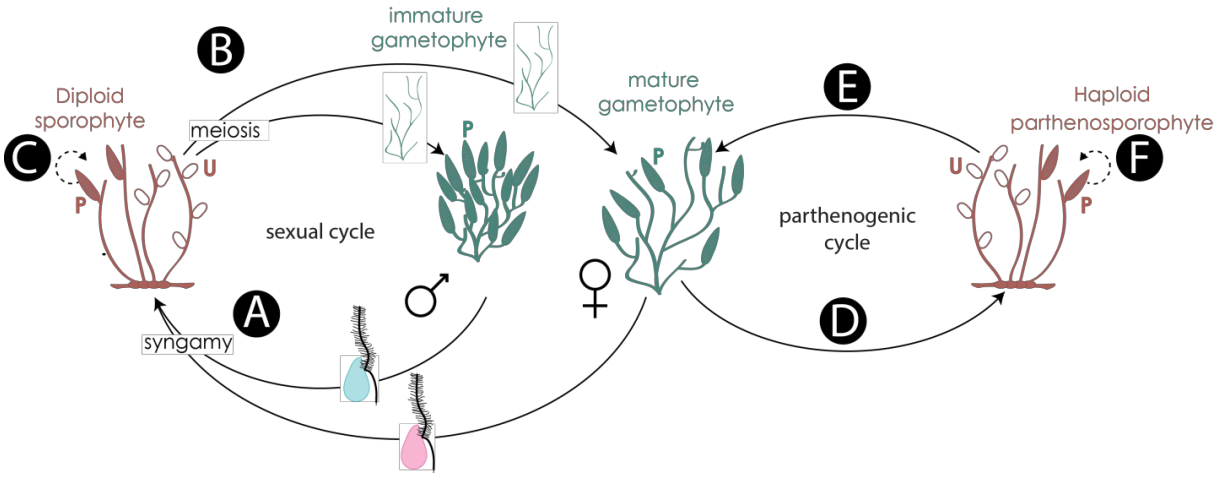


Figure 5. ***Ectocarpus*** **life cycle**. U: Unilocular sporangia; P: Plurilocular sporangia on the sporophyte and partheno-sporophyte stages; Plurilocular gametangia on gametophytes. See text for details.

Objectives

The general aim of this thesis was to gain insights into the molecular, genetic and evolutionary mechanisms of sex determination and differentiation in the brown alga *Ectocarpus*. This PhD work analysed key features of sexual reproduction in *Ectocarpus*, from the identification and characterization of sex chromosomes to the genetic and cellular basis of sexual dimorphisms. More specifically the objectives of my PhD were:

1. To perform a functional and evolutionary analysis of the sex chromosome of *Ectocarpus*, specifically to study the expression of the SDR genes during the haploid-diploid life cycle of *Ectocarpus* and to investigate the evolutionary features of the pseudoautosomal regions in the sex chromosome. This will be described in Chapter 2 and 3.
2. To identify sexual dimorphisms and analyse the cascade of gene expression that is involved in sexual differentiation in *Ectocarpus* (Chapter 4).
3. To investigate the genetic relationship between the sex chromosome and parthenogenesis, which is a sexually dimorphic trait in some *Ectocarpus* accessions. (Chapter 5).
4. To investigate the cellular basis of early parthenogenetic development (Chapter 6).

Chapter 2. The Haploid System of Sex Determination in the Brown Alga *Ectocarpus* sp.

I. Introduction

Dimorphic sex chromosomes have evolved independently from autosomes in many taxa, ranging from animals to plants (Bull 1983; Charlesworth 1990, 1996; Rice 1996). Factors such as the rate of mutation and recombination, as well as intra-genomic conflict, play a pivotal role in the evolution of sex-determining regions and sex chromosomes. Once reduced recombination of the sex chromosomes has evolved, the non-recombining region gradually decays due to the accumulation of deleterious mutations (*e.g*. Bergero and Charlesworth, 2009; Gordo and Charlesworth, 2001). These processes have been widely studied in theory (*e.g*. Charlesworth and Charlesworth, 1978; Rice, 1987) and empirically for diploid organisms with XY or ZW systems (*e.g* Bergero and Charlesworth, 2009; Handley *et al*., 2004; Matsubara *et al*., 2006; see Bachtrog, 2013 for review) However, many lineages, particularly protists, fungi, and plants, spend an important portion of their life as haploids (Mable and Otto, 1998). In species with an independently-living multicellular haploid phase, it is the haploid phase (the "gametophyte") that exhibits male and female sexual organs. The diploid phase in these organisms (the "sporophyte") lacks sexual differentiation and reproduces asexually via mitotic spores or sexually via meiotically-produced spores. It has been proposed that sex chromosomes in organisms with this type of haploid-diploid life cycle (UV systems) evolve differently compared with diploid sex chromosomes (Bull, 1978).

Both U and V sex chromosomes are non-recombining, and have an effective population size that is half that of the autosomes. The strength of selection is expected to be reduced in U and V sex chromosomes, which, as in diploid systems, should induce genetic degeneration of U and V. However, because these chromosomes function during the haploid phase of the life cycle they should experience purifying selection, which should counteract the degenerative effects. Therefore, genes that are important for the haploid phase should not degenerate. On the other hand, any genes on either the U or V that are expressed during the diploid sporophyte phase will be sheltered and hence both U and V chromosomes may potentially degenerate (Lewis, 1961; Lewis and John, 1968). Some signs of genetic degeneration were indeed found in the few UV systems studied so far. In the bryophyte

Marchantia, analysis of the U chromosome demonstrated a reduction in gene density and an increase in TE density compare to the autosomes (Yamato *et al*., 2007). Similarly in *Volvox*, the non-recombining mating-type locus was shown to be gene poor and rich in repeated sequences but also to exhibit a decrease in codon usage bias compare to autosomes (Ferris *et al*., 2010). Another verbal prediction that was made for UV systems is that the expansion and evolution of the non-recombining region is more likely to be due to the addition of genetic material instead of gene loss. The former could be advantageous if the segment that is moved into the SDR is carrying some genes that are favourable for one of the two sexes (Bull, 1978). In *Chlamydomonas*, at least two events of translocation or duplication with an autosomal origin were found in the locus mating-type (MT) *plus*, which is consistent with the idea proposed by Bull (Ferris *et al*., 2002).

The apparent symmetry of the UV sex chromosomes' life history led Bull (1978) to hypothesize that U and V should evolve symmetrically and that any degeneration should be equal in both U and V sex chromosomes. However the selective pressure that each sex experiences is often different, with males more exposed to sexual selection than females (see Chapter 1). This could result in asymmetric evolution of the U and V.

We took advantage of the tools that have been developed for the model brown alga *Ectocarpus* in recent years, including genome sequences of both male and female strains (*Ectocarpus* 1c lineage, Stache-Crain *et al*., 1997), to identify and perform an evolutionary and functional analysis of the sex-determining region of this organism.

II. Paper

Title:

A Haploid System of Sex Determination in the Brown Alga *Ectocarpus* sp.

(Article published in Current Biology)

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R.L. contributed to this paper by preparing the *Ectocarpus* cultures, performing the expression analyses, identifying sex-specific scaffolds and sex-markers in different *Ectocarpus* strains.

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Article

A Haploid System of Sex Determination in the Brown Alga *Ectocarpus* sp.

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Summary

Background: A common feature of most genetic sex-determination systems studied so far is that sex is determined by nonrecombining genomic regions, which can be of various sizes depending on the species. These regions have evolved independently and repeatedly across diverse groups. A number of such sex-determining regions (SDRs) have been studied in animals, plants, and fungi, but very little is known about the evolution of sexes in other eukaryotic lineages.
Results: We report here the sequencing and genomic analysis of the SDR of *Ectocarpus*, a brown alga that has been evolving independently from plants, animals, and fungi for over one giga-annum. In *Ectocarpus*, sex is expressed during the haploid phase of the life cycle, and both the female (U) and the male (V) sex chromosomes contain nonrecombining regions. The U and V of this species have been diverging for more than 70 mega-annum, yet gene degeneration has been modest, and the SDR is relatively small, with no evidence for evolutionary strata. These features may be explained by the occurrence of strong purifying selection during the haploid phase of the life cycle and the low level of sexual dimorphism. V is dominant over U, suggesting that femaleness may be the default state, adopted when the male haplotype is absent.
Conclusions: The *Ectocarpus* UV system has clearly had a distinct evolutionary trajectory not only to the well-studied XY and ZW systems but also to the UV systems described so far. Nonetheless, some striking similarities exist, indicating remarkable universality of the underlying processes shaping sex chromosome evolution across distant lineages.

Introduction

Genetic determination of sex is mediated by sex-determining regions (SDRs) of various sizes or by sex chromosomes in a broad range of eukaryotes. Sex chromosomes have arisen independently and repeatedly across the eukaryotic tree, and comparative analysis of different sex-determination systems has provided insights into how these systems originate and evolve. A typical sex chromosome pair is thought to have derived from a pair of autosomes through the acquisition of genes involved in sex determination. If more than one locus involved in sex determination is located on the chromosome, recombination between loci is expected to be suppressed to avoid the production of maladapted individuals with a combination of male and female alleles of the sex-determining genes. This leads to the establishment of a nonrecombining region on the nascent sex chromosome, with important consequences for the evolution of this region of the genome [1]. For example, as a result of the suppression of recombination within the SDR, repetitive DNA tends to accumulate, leading to an increase in SDR size and degeneration of genes within the nonrecombining region. At a later stage, deletion of nonfunctional DNA from within the SDR may lead to a decrease in the physical size of the SDR.

There is also evidence that the nonrecombining region can progressively encroach on the flanking regions of the chromosome so that it encompasses an increasingly greater proportion of the sex chromosome. This process is thought to be driven by the recruitment of genes with differential selective benefits to the two sexes (sexually antagonistic genes) into the SDR [2] (but see [3]). Extension of the SDR in this manner can lead to the creation of "strata," which are regions of the SDR that have become nonrecombining at different points in evolutionary time [4–7].

The genetic mechanism of sex determination also influences how the sex chromosomes evolve. In organisms in which sex is expressed in the diploid phase, such as most animals and land plants, one sex is heterogametic (XY or ZW), whereas the other is homogametic (XX or ZZ). In these systems, only the Y or W contains nonrecombining regions because the X and Z recombine in the homogametic sex. In some algae and bryophytes, the male and female sexes are genetically determined after meiosis, during the haploid phase of the life cycle [8, 9]. This type of sexual system, termed UV to distinguish it from the XY and ZW systems described above [10], exhibits specific evolutionary and genetic properties that have no exact equivalent in diploid systems. In UV systems, the female and male SDR haplotypes function in independent, haploid, male and female individuals, and, consequently, there is no heterozygous sex comparable to XY males or ZW females. This difference between UV and XY/ZW systems should have important implications for SDR evolution [8, 9]. In particular, the female U and the male V are expected to be under similar evolutionary pressures not only because they function

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independently in different individuals but also because neither the U nor the V SDR haplotype recombines [8, 9]. As a result, both haplotypes are expected to exhibit the effects of loss of recombination, such as gene degeneration, to a similar extent. Gene degeneration is, however, expected to be limited in both the U and the V regions, provided they both contain genes that are essential during the haploid phase. It has also been suggested that changes in the size of the U or V involved principally additions of beneficial (but not essential) genes rather than gene losses [8, 9]. Some asymmetry may be expected between the U and V, however, if sexual selection is stronger in males [11] or if one of the chromosomes plays a more active role in sex determination. These verbal predictions of the characteristics of UV systems still need to be rigorously tested empirically.

Although eukaryotic species with UV systems may be as common as those with XY and ZW systems, very few of the former have been characterized, with detailed sequence data being available for only two members of the Archaeplastida lineage: the liverwort *Marchantia* (which has a fully sequenced V chromosome but a partially identified U chromosome) [12] and a UV pair of unknown age in the green alga *Volvox* [13], together with more fragmentary information recently obtained for the moss *Ceratodon* [14]. Clearly, additional detailed sequence information is required to fully test the predictions that have been made with respect to UV sex-determination systems and to evaluate the generality of these predictions in a broad phylogenetic context.

We report here the identification and the genetic and genomic characterization of the U and V sex-determining regions of the brown algal model *Ectocarpus* sp. (formerly included in *E. siliculosus*) [15, 16]. Brown algae belong to the Stramenopiles, a lineage very distantly related to animals, fungi, and green plants (the common ancestors dating back more than one giga-annum [Ga]). The brown algae are considered to possess sex chromosomes rather than mating-type chromosomes [17–19] for a number of reasons: (1) there is a strict correlation between gamete size and sex in anisogamous species; (2) all sexual brown algal species exhibit some form of sexual dimorphism [20, 21]; and (3) heteromorphic sex chromosomes have been identified in some species [22, 23]. Previous work has shown that sex is determined by a single, Mendelian locus in *Ectocarpus* sp. [24]. During the haploid-diploid life cycle of this organism, meiospores, produced by the sporophyte generation, develop into dioicous (separate male and female) gametophytes, which then produce either male or female anisogametes (Figure 1A).

We show here that the *Ectocarpus* sp. UV has features typical of sex chromosomes in other systems, such as low gene density and a large amount of repeated DNA. The male and female SDRs are extremely diverged, reflecting a long independent evolutionary history, which we estimated at more than 70 mega-annum (Ma). Despite its age, the SDR constitutes only one-fifth of the sex chromosome. A possible explanation for this observation was suggested by the low number of sex-biased genes, implying that sexual conflict may be insufficient in *Ectocarpus* sp. to drive extensive SDR expansion. Both the male and female SDR haplotypes showed signs of degeneration despite the action of purifying selection during the haploid phase of the life cycle. Analysis of expression data suggested that the genes escaped degeneration function during the haploid phase of the life cycle. The male SDR haplotype was dominant over the female haplotype, suggesting that the V chromosome determines maleness, with femaleness possibly being the default state when this chromosome is absent. A male-specific high mobility group (HMG) domain gene was identified as a candidate male sex-determining gene. Analysis of the *Ectocarpus* sp. SDR has underlined the universality of sex chromosome evolution across the eukaryotes and has provided important insights into sex chromosome evolution in UV sexual systems.

Results

Identification and Characterization of the *Ectocarpus* sp. SDR

The initial screen for SDR sequence scaffolds used comparative genome hybridization experiments [25] to identify three male-specific scaffolds. PCR-based markers were used to localize these scaffolds to linkage group 30 of the *Ectocarpus* sp. genetic map [26] (Figure 1B; Tables S1A–S1C available online). Searches for additional male SDR scaffolds were then carried out by searching for scaffolds carrying male-specific genes using male and female transcriptomic data and by adapting the Y chromosome genome scan (YGS) method, which uses short-read sequencing and k-mer comparison to identify sex-linked sequences [27] (see the Supplemental Experimental Procedures for further details). Together, these methods allowed the identification of two large sequence scaffolds corresponding to the male SDR haplotype. Sex linkage was systematically verified by genetic mapping (Tables S1B and S1C).

Further analysis of the segregation patterns of genetic markers corresponding to SDR scaffolds in a single family of 2,000 siblings detected no recombination events (Figure 1B). The SDR therefore behaves as a discrete, nonrecombining haplotype. This genetic analysis indicated that the male SDR extended over a region of approximately 920 kilobase pairs (kbp) (Figure 1C; Table 1).

To characterize the female haplotype of the sex locus, we sequenced the genome of a female *Ectocarpus* sp. strain that is closely related to the sequenced male strain (Figure S1A) [16]. Several strategies were used to identify candidate female SDR scaffolds (Supplemental Experimental Procedures and Tables S1E–S1H). These included searches for female orthologs of male SDR protein sequences, a search for scaffolds carrying female-specific genes based on male and female transcriptomic data, and the adaptation of the YGS method [27] to search for female rather than male scaffolds. The cumulative size of the female sex-linked scaffolds was 929 kbp. Assuming that the combination of approaches used here has provided a near-complete list of male and female SDR scaffolds, this indicates that the male and female SDR haplotypes are of similar size (Figure 1C; Table 1).

To confirm cosegregation of the SDR with sexual phenotype, 34 *Ectocarpus* strains of known sex from different geographical origins and species were genotyped with several sex locus markers, corresponding to both the male and female SDR haplotypes (Table S1D). In all cases, the SDR genotype correlated with sexual phenotype, confirming that this region is the sex-determining locus in *Ectocarpus*.

The SDR is flanked by two large recombining regions, which we refer to as pseudoautosomal (PAR) domains. Analysis of molecular marker segregation [26] indicates that these regions recombine during meiosis, unlike the SDR (Figure 1B). The PAR had gene density, intron length, and percent GC content intermediate between those of the autosomes and the SDR (Figure 1B; Table 1). These unusual features are characteristic

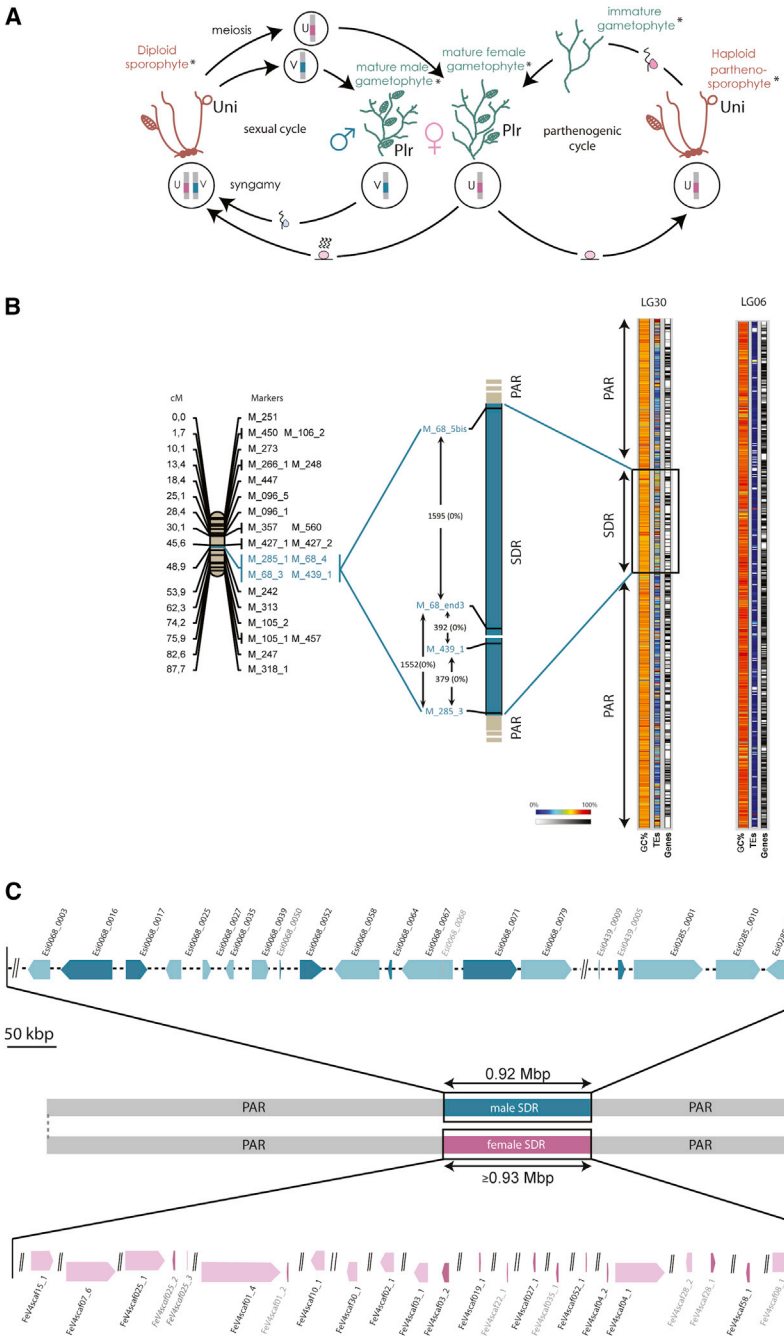

Figure 1. The UV Sex-Determination System of the Brown Alga *Ectocarpus* sp.

(A) Life cycle of *Ectocarpus* sp. in culture. The sexual cycle (left side of panel) involves an alternation between the diploid sporophyte and haploid, dioicous (male and female) gametophytes. The sporophyte produces meiospores through meiosis in unilocular sporangia (single-chambered, spore-bearing structures; Uni). The meiospores are released and develop as gametophytes (each containing either a U or a V sex chromosome), which then produce gametes in plurilocular gametangia (multiple-chambered, gamete-bearing structures; Plr). Fusion of male and female gametes produces a zygote (containing both the U and the V sex chromosomes), which develops as a diploid sporophyte, completing the sexual cycle. Unfertilized gametes can enter an asexual parthenogenetic cycle by germinating without fusion to produce a partheno-sporophyte (right side of panel). The partheno-sporophyte produces spores through apomeiosis in unilocular sporangia, and these develop as gametophytes, completing the parthenogenetic cycle. Note that the haploid partheno-sporophytes and the diploid sporophytes do not express sex. The parthenogenetic cycle is only shown for a female, but male gametes can also develop parthenogenetically in some *Ectocarpus* lineages. Life cycle stages used for the qRT-PCR analysis of SDR gene expression are marked with an asterisk.

(B) Genetic and physical maps of the *Ectocarpus* sp. sex chromosome. The left side of the panel shows a genetic map of the *Ectocarpus* sp. sex chromosome (LG30). The positions of simple sequence repeat (SSR) markers are indicated to the right of the linkage group, with the prefix "M" for marker, followed by the number of the supercontig that contains the SSR, and, finally, in some cases, with a suffix to distinguish markers that originated from the same supercontig. Sex-linked markers are shown in blue. Numbers to the left indicate map distances (in cM) between the intervals given by the lines that cross the vertical bar. The genetic map was generated using a segregating family of 60 individuals, except for the nonrecombining region, where a larger population of 2,000 meiotic individuals was used. The central panel depicts the extent of recombination between markers located inside the *Ectocarpus* sp. nonrecombining region. The number of meiotic siblings used to assay for recombination between each pair of markers is indicated, with the percentage of recombinants detected in parentheses. Note that no recombination was detected between any of the sex locus markers. See Table S1B for the coordinate position of each marker on its respective scaffold. The right side of the panel shows a physical map of the sex chromosome and a heatmap of the GC percent, gene density, and TE density along the LG30 and along an autosome (LG06) for comparison. The heatmap was computed using a 4,000 base pair (bp) sliding window.

(C) Overview of the *Ectocarpus* sp. male and female SDR haplotypes. Genes are indicated by arrows, with the lighter colors corresponding to gametologs. Gene names (LocusIDs) are indicated, with pseudogenes in gray font and putative transposon remnants in gray italics. Putative transposon remnants were counted as protein-coding genes, but Esi0068_0068/FeV4scaf25_3 was not included in the set of gametolog pairs. The relative sizes of the male and female SDR genes are indicated, but they are not drawn to the same scale as the underlying scaffolds indicated by the dotted line and the scale bar. Only female SDR scaffolds carrying genes are represented. Scaffolds are separated by double diagonal lines, indicating that the relative positions of scaffolds within the SDR are unknown. Double-headed arrows indicate the estimated sizes of the SDR haplotypes. The gray bars indicate the sex chromosomes. SDR, sex-determining region; PAR, pseudoautosomal region. See also Figure S1.

of the entire recombining part of the chromosome and are not restricted to the regions closest to the SDR (Figure 1B). It is currently not clear why the PAR exhibits these structural differences compared to the autosomes.

Both the male and female SDR haplotypes are rich in transposable element sequences (Figure 1B; Figure 2A) and gene poor compared to the autosomes (Table 1), features typical of nonrecombining regions [1]. With only one exception (long

Table 1. Statistics for Several Features of the Male and Female *Ectocarpus* sp. SDR Compared with the PAR and the Complete Genome

	Male SDR	Female SDR	PAR	Genome
Total sequence (Mbp)	0.92	0.93	4.08	205.27
Genes (including pseudogenes)	20	24	228	15,779
Average gene length (bp)	25,710	18,836	8,188	6,974
Average CDS length (bp)	1,373	1,050	1,217	1,607
Average intron length (bp)	3,605	3,691	1,062	702
Average number of introns per gene	6.67	4.81	6.28	7.14
Gene density (genes per Mbp)	22.82	23.66	55.88	76.87
GC (%)	51.29	44.74	52.20	54.02

terminal repeat transposons in the female SDR), all transposable element (TE) classes were more abundant in the SDR and the PAR than they were in the autosomes, with the differences being particularly marked for both SDR haplotypes. When individual classes of transposable elements were considered, retrotransposons (which represent the least abundant transposon class in the *Ectocarpus* sp. genome as a whole) showed the most marked proportional enrichment in the SDR haplotypes compared to the autosomes (Figure S2A).

About 30% of the euchromatin of the male-specific (nonrecombining) region of the human Y chromosome consists of multiple, different "ampliconic sequences," which exhibit 99.9% identity within each set of repeated sequence. The identity between these sequences has been taken as evidence for a high level of gene conversion within this region [5, 30]. It was further suggested that gene conversion might "substitute" for interchromosomal recombination to some extent, counteracting the degenerative effects of reduced recombination within the SDR. Very little intrahaplotype sequence similarity was identified within either the male or the female *Ectocarpus* sp. SDR haplotypes (Table S1J). The total lengths of the repeated regions within the male and female SDRs were only 2.5% and 3.2%, respectively. It therefore seems unlikely that mechanisms similar to those proposed for the human Y chromosome have operated in this SDR, although it should be noted that large ampliconic repeats are difficult to assemble, and some sequences of this type may not have been identified, particularly for the female haplotype.

The male SDR haplotype contains 17 protein-coding genes and three pseudogenes, whereas 15 protein-coding genes and seven pseudogenes were found in the female haplotype (Figure 1C; Figure 3; Table S2). Eight of the female protein-coding genes and three of the pseudogenes are homologous to male SDR sequences ("gametologs"), consistent with the two SDR haplotypes having evolved from a common ancestral autosomal region. The classification of these genes as gametologs was supported by expression analysis, which showed that transcript abundances for gametolog pairs were strongly correlated (Figure S2B), and by their conserved intron and exon structures (Figure S3). This correlated expression pattern is consistent with the gametolog genes having been retained because they have non-sex-specific functions during the haploid phase of the life cycle. The genes and pseudogenes that were only found in one (male or female) haplotype may have been either acquired since the divergence of the U and the V regions or lost by the counterpart haplotype. Eighteen of the male and female genes and pseudogenes that were found in only one haplotype had homologs outside the SDR (including, in two cases, genes on linkage group 30; Figure 3 and Table S2). The high similarity between some of these SDR genes and their closest autosomal homologs would be consistent with these gene pairs having arisen from recent gene duplication events (i.e., since the divergence of the U and the V) that created either the SDR or the autosomal copy. The remaining two genes that were found in only one haplotype may represent cases of gene loss in the other haplotype, but they could also have resulted from gene relocation to the SDR. Testing these hypotheses will require comparison with a homologous gene from an outgroup species.

Genomic Degeneration of the SDR Region

Suppression of recombination across the SDR is expected to lead to genetic degeneration unless there is strong selection on gene function to counteract this effect. There are several indications that genetic degradation has occurred, at least to some degree, in the *Ectocarpus* sp. SDR. We identified a set of optimal codons for *Ectocarpus* sp. (Figures S2C and S2D). Selection on codon usage is known to be of weak intensity and particularly sensitive to loss of recombination [31, 32]. The coding sequences of SDR genes exhibited significant underrepresentation of optimal codons (Figure 2B). This suggests maladapted codon usage (although we cannot exclude that the underrepresentation is due, at least in part, to reduced rates of biased gene conversion [33] due to the loss of recombination within the SDR). In addition, transcripts of SDR genes tended to be less abundant on average than transcripts of autosomal genes, although note that codon usage and expression level are likely to be correlated, so these two parameters are not necessarily independent. Reduced transcript abundance was particularly marked for SDR genes that were exclusively present in one of the haplotypes (Figure 2C), and it may reflect degradation of the promoter and *cis*-regulatory sequences of these SDR genes. The same tendency was observed for the *Volvox* mating locus, where haplotype-specific genes were expressed at lower levels than genes that were part of a gametolog pair [13], suggesting that genetic degeneration of haplotype-specific SDR genes may be a general phenomenon. Note that expression analysis of the *Ectocarpus* sp. gametolog genes did not provide any evidence that these genes are degenerating.

SDR genes were found to be much longer on average than genes elsewhere in the genome, due principally to the presence of longer introns (Table 1). This difference was partly explained by the presence of a larger amount of inserted transposable element DNA (Figures 2A and S2E), which is typical of nonrecombining regions.

Although these various analyses provided some evidence for genomic degeneration in the SDR, the overall degree of degeneration was modest compared to previously characterized systems [34], perhaps because both the U and the V SDR haplotypes have essential functions during the haploid phase and are constantly exposed to selection (in contrast to Y or W chromosome genes, which are always heterozygous). An analysis of SDR gene expression supported this hypothesis: transcripts of SDR genes were consistently present during the haploid phase of the life cycle (Figure 4). Another potential explanation for the limited degree of degeneration is that the SDR is small compared to most previously characterized systems, and this may have limited the potential for Hill-Robertson interference among selected sites [35–37].

Predicted Functions of SDR Genes

Of the nine genes that were found in the male, but not the female, SDR haplotype, one was of particular interest because

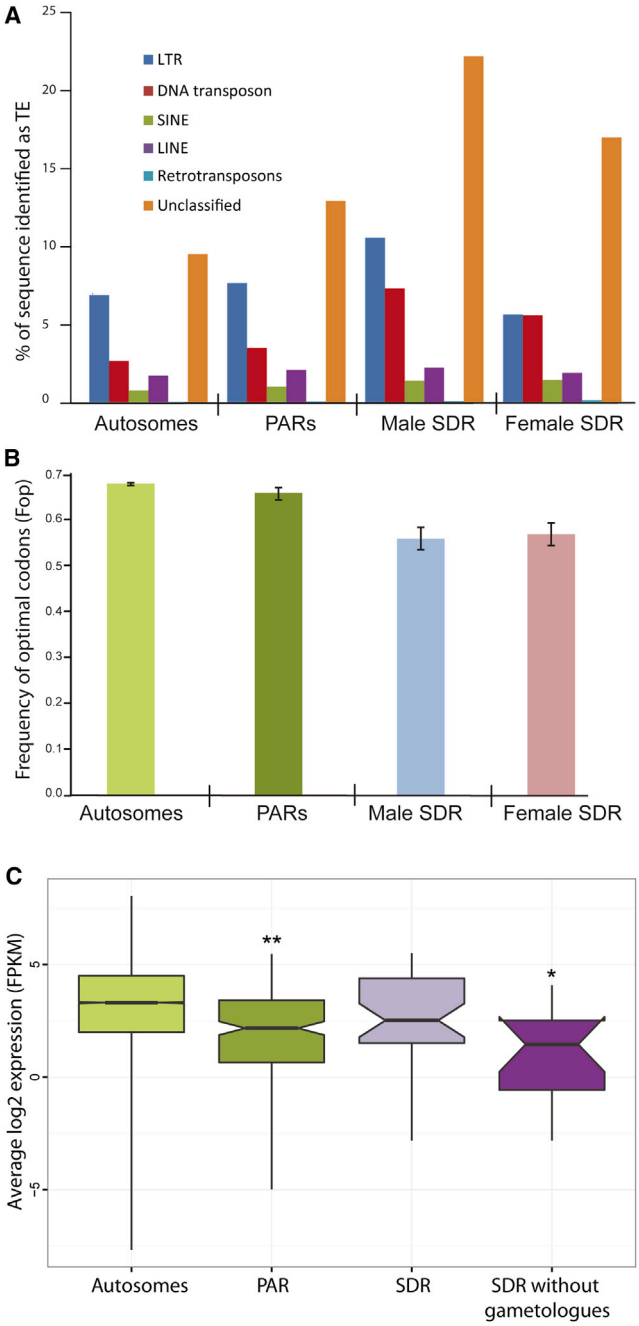

Figure 2. Comparison of Genomic Features of the SDR, PAR, and Autosomes

(A) Percentage of DNA corresponding to different classes of transposable elements (TEs) in different genomic fractions. Pairwise comparisons using a Fisher's exact test indicated that all of the sex chromosome compartments (PAR, male SDR, female SDR) were significantly different from the autosomal compartment ($p < 0.0001$).

(B) Median frequency of optimal codons in coding regions of autosomal, PAR, and male and female SDR genes. Error bars indicate 95% confidence intervals around the median. An analysis using the codon adaptation index (CAI, another codon usage index [28], which was computed using R and the seqinR package [29]) gave similar results.

(C) Mean transcript abundance in sexually mature, male and female gametophytes for genes in different genome fractions, determined by RNA-seq and expressed as fragments per kilobase per million reads (FPKM) mapped. The notched boxplot graph shows the means of autosomal genes (n = 14,677), PAR genes (n = 205), male and female SDR genes (n = 37), and SDR without gametolog genes (n = 16). Significant adjusted p values compared with autosomes, as calculated by Wilcoxon tests, are indicated by asterisks above each box (*$p < 0.01$, **$p < 0.001$).

it was predicted to encode an HMG domain protein (Figure S4A and Table S4A). This family of proteins has been implicated in sex or mating-type determination in both vertebrates and fungi [38, 39]. The SDR of the green alga *Volvox* also contains an HMG gene [13]. In addition, several of the genes that were found in both the male and female SDR haplotypes (gametologs) were predicted to encode potential signal transduction proteins (including a Ste20-like kinase, a casein kinase, a GTPase, a RING zinc-finger protein, and a MEMO domain protein; Table S2) and could potentially be involved in the regulation of sex determination.

An Ancient Sex-Determining Region

At the sequence level, the male and female haplotypes are extremely divergent. No large blocks of sequence similarity were found, and the only regions with a high level of similarity corresponded to gametolog exons (Figure S3). This divergence suggests that the male and female haplotypes have been evolving independently over a long period. Two phylogenetic trees were constructed based on sequences of either an SDR or an autosomal sequence from three *Ectocarpus* lineages and three distantly related brown algal species, *Scytosiphon lomentaria*, *Sphaerotrichia firma*, and *Laminaria digitata*. The topology of the phylogenetic tree based on the autosomal region was consistent with sequential speciation, with sequences from male and female strains of the same lineage grouping together (Figure 5A). In contrast, in the phylogenetic tree based on the SDR gene, sequences grouped together according to gender (Figure 5B). Note that we were not able to obtain sequence for this gene from female *L. digitata* individuals, suggesting that they may have lost the female gametolog. These data suggest that the SDR originated at least 70 million years ago and may be substantially older. The rate of synonymous site mutations (dS) in the coding regions of the 11 male and female gametolog pairs (Figure 5C) was used to independently evaluate the age of the SDR. The dS values for these gene pairs were compared with values for orthologous, autosomal gene pairs across 12 brown algal and diatom species for which divergence times had been estimated (Supplemental Experimental Procedures). The dS values for the SDR genes were remarkably high (mean value of 1.7, with most genes having $dS > 1$), and comparisons with values obtained for the pairs of autosomal orthologs indicated that the male and female haplotypes of the SDR stopped recombining more than 100 million years ago (Figure S5). Note, however, that the estimations based on genetic divergence are approximate because of saturation of synonymous site mutations at the evolutionary distances measured. These analyses suggest that the *Ectocarpus* sp. UV SDR is an old system, comparable to the *Drosophila* (60 Ma) [34] and mammalian (180 Ma) [41, 42] XY systems.

When dS values were calculated on an exon-by-exon basis, individual exons with a markedly lower dS value than those of the other exons within the gametolog gene pair were identified for 3 of the 11 gametolog pairs (Figure S3). The presence of these rare variant exon pairs suggests that gene conversion events affecting individual exons or small gene regions may have occurred since the divergence of the male and female SDR haplotypes, but more detailed studies are needed to address this possibility.

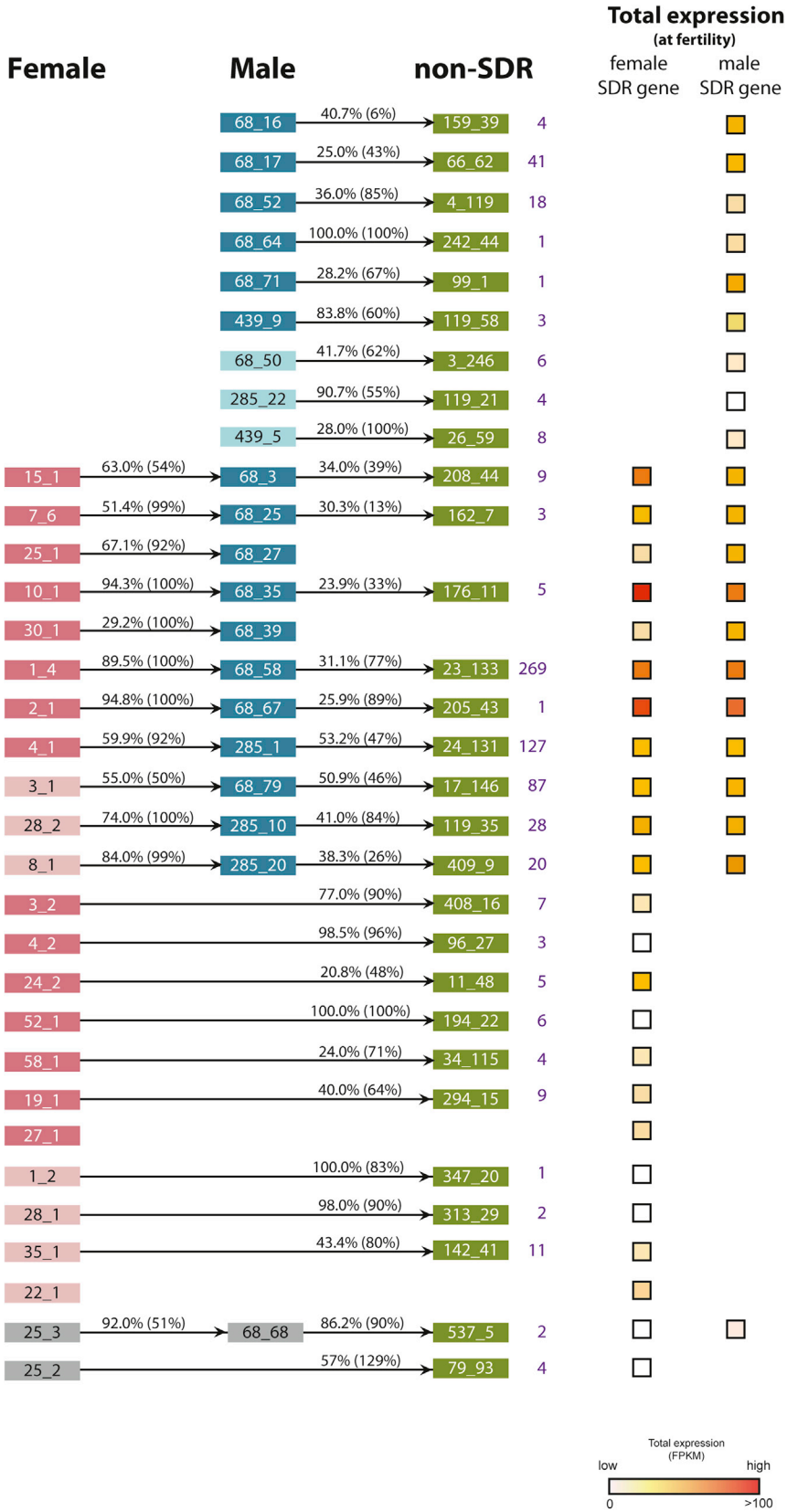

Figure 3. Relationships between SDR Genes and Autosomal Genes and Expression Patterns of the SDR Genes

Schematic diagram showing homology relationships between male and female SDR genes and autosomal genes. Autosomal or PAR (i.e., non-SDR) genes are shown in green; male and female SDR genes are shown in blue and pink, respectively, with putative functional genes in dark blue or dark pink and pseudogenes in light blue or light pink. Putative transposon remnants are shown in gray. A green box indicates the existence of at least one homolog outside the SDR, and the number to the right of the green box indicates the number of matches outside the SDR with an E value of less than 10^{-4}. Homology relationships were defined based on a BLASTP E value of less than 10^{-4} when predicted protein sequences were blasted against the complete set of *Ectocarpus* sp. predicted proteins. Percentage identity between predicted proteins is indicated above the arrows. The value in parentheses corresponds to the length of the matched region as a percentage of the total length of the protein to the left of the arrow. Gene abbreviations are as in the following examples: for male SDR or non-SDR genes, 68_16 indicates Esi0068_0016; for female SDR genes, 15_1 indicates FeV4scaf15_1. Note that the order of the genes is not intended to correspond to their locations in the genome. The right side of the panel depicts transcript abundances for each of the male and female SDR genes in male and female mature gametophytes, respectively, measured by RNA-seq and expressed as FPKM. See also Figure S2.

Limited Expansion of the *Ectocarpus* sp. SDR

Given its age and the prediction that an SDR should progressively enlarge over time to encompass a large part of its chromosome [1, 43], it is remarkable that the *Ectocarpus* sp. SDR accounts for only about one-fifth of linkage group 30 and extends over less than one megabase pair (Mbp). It is possible that the small size of the SDR is related to the low level of sexual dimorphism in *Ectocarpus* sp. because the recruitment of sexually antagonistic genes is believed to be an important driver of SDR expansion [1, 43]. Moreover, sexually antagonistic polymorphisms are predicted to be less stable in haploid systems than in diploid systems because dominance effects in XX (or ZZ) individuals are expected to favor allele maintenance in the latter [44, 45]. This effect may also limit expansion of the SDR by reducing the number of genes with sexually antagonistic polymorphisms available for recruitment into the SDR. Consistent with these hypotheses, comparison of the transcriptomes of male and female gametophytes indicated that only about 4% of *Ectocarpus* sp. genes showed sex-biased expression at the mature sexual stage of the life cycle (compared, for example, with up to 50%–75% in *Drosophila* [46, 47]; Table S4C).

SDR Gene Expression and Dominance

Quantitative PCR was used to measure the abundance of SDR gene transcripts in near-isogenic male and female strains (Figure 4) at different stages of the life cycle (Figure 1A). Whereas no clear pattern was observed for the female SDR genes, transcripts of two-thirds of the male SDR genes that were analyzed were most abundant in mature gametophytes (Figure 4), suggesting that these genes have a role in fertility. Interestingly, the transcript of the male gene that is predicted to encode an HMG domain protein was more than 10-fold more abundant in mature gametophytes than at the other stages assayed (Figure 4). The other fertility-induced genes included both additional male-specific genes (encoding conserved unknown proteins) and several gametolog pairs (predicted to encode, for example, a GTPase, a MEMO-like domain protein, a nucleotide transferase, and a homoaconitate hydratase; Table S2).

Diploid gametophytes bearing both the male and the female SDR haplotypes (UV) can be generated artificially, and these individuals are always phenotypically male, indicating that the male haplotype is dominant [24, 48]. This dominance relationship would be consistent with the existence of a master regulatory gene that determines maleness, carried by the V chromosome. To determine whether the dominance of the male haplotype is dose dependent, we used the life cycle mutant *ouroboros* [48] to construct 13 independent triploid (UUV) and tetraploid (UUUV) gametophytes (Figure S1A and Table S1I). All tested polyploids produced male gametes (as determined by genetic crosses with tester lines). Measurements of transcript abundances for 11 female SDR genes did not detect a marked downregulation of these genes in diploid heterozygous gametophytes compared to haploid gametophytes (Figures S4B and S4C). This suggests that the male haplotype does not silence female gene expression in this heterozygous context (although it was not possible to rule out that the expression of specific female haplotype genes was suppressed). It is likely, therefore, that gametophytes adopt the female developmental program by default, when the male SDR haplotype is absent.

Discussion

This study has demonstrated that sex is determined during the haploid phase of the brown alga *Ectocarpus* sp. by a nonrecombining region on linkage group 30 that extends over almost 1 Mbp. The male and female haplotypes of the SDR were of similar size but were highly diverged, with the only significant similarity being the presence of 11 gametologs, three of which were predicted to be pseudogenes in the female. Based on comparisons of these shared genes across diverse brown algal species, the SDR was estimated to be more than 100 million years old. Compared with previously characterized systems [49], the *Ectocarpus* sp. UV chromosomes can clearly be classed as an ancient (as opposed to a recently evolved) sex-determining system.

The brown algae belong to the Stramenopiles, which diverged from the lineages that led to green plants and animals more than one billion years ago [50]. This study therefore confirms that SDRs from diverse eukaryote groups share a number of fundamental features, such as stable maintenance of pairs of functional alleles (gametologs) over long periods of evolutionary time, suppressed recombination within the SDR, low gene density, and accumulation of transposable elements. The presence of 11 gametolog pairs provided unambiguous evidence that the *Ectocarpus* sp. UV pair is derived from an ancestral pair of autosomes, as has been observed for XY and ZW systems in animals and plants [1, 7, 43].

Analysis of the *Ectocarpus* sp. SDR has also allowed a number of predictions that specifically concern UV sexual systems [8, 9] to be tested. UV systems are not expected to exhibit the asymmetrical degeneracy of the sexual chromosomes (degeneracy of the Y and W chromosomes) observed in XY and ZW systems [34], and this supposition is supported by the similar estimated sizes of the male and female SDR haplotypes in *Ectocarpus* sp. Based on parameters such as transcript abundance and frequency of optimal codons, the *Ectocarpus* sp. SDR genes exhibit evidence of degeneration, but the degree of degeneration is modest compared to that observed for Y-located genes in XY systems of comparable age [34]. Because transcripts of all the SDR genes were detected in the gametophyte generation, the modest degree of degeneration is consistent with purifying selection acting to maintain gene functionality during the haploid phase, when the U and V chromosomes are found in separate male and female organisms. Selection is indeed expected to be stronger during the haploid phase, and it is expected to limit degeneration, as suggested for the V chromosome of *Marchantia* [12], another UV system, and by the low nonsynonymous to synonymous site mutation (dN/dS) ratios observed for sex-linked pollen-expressed genes in *Silene latifolia*, a plant with XY chromosomes [51]. The detection of modest levels of gene degeneration indicates that UV SDRs are nonetheless subject to the degenerating effects of suppressed recombination to some degree. Expression analysis indicated that in *Ectocarpus* sp., the SDR genes that escape degeneration belong principally to gametolog pairs, which presumably play a role during the haploid phase, or are male haplotype-specific genes, which are presumably required for male fertility. The *Ectocarpus* sp. SDR contains a large proportion of sex-specific genes (20 male and female sex-specific genes compared with only 11 gametolog pairs). This situation contrasts markedly with the UV system of *Volvox*, where the vast majority of the mating region genes are shared between haplotypes [13]. This difference in gene composition suggests that these two UV systems have had different evolutionary histories, perhaps having been affected in different ways by gene gain and gene loss events. Bull predicted that changes in the sizes of the U and V SDR haplotypes should be due to gain of genes beneficial to the gametophyte

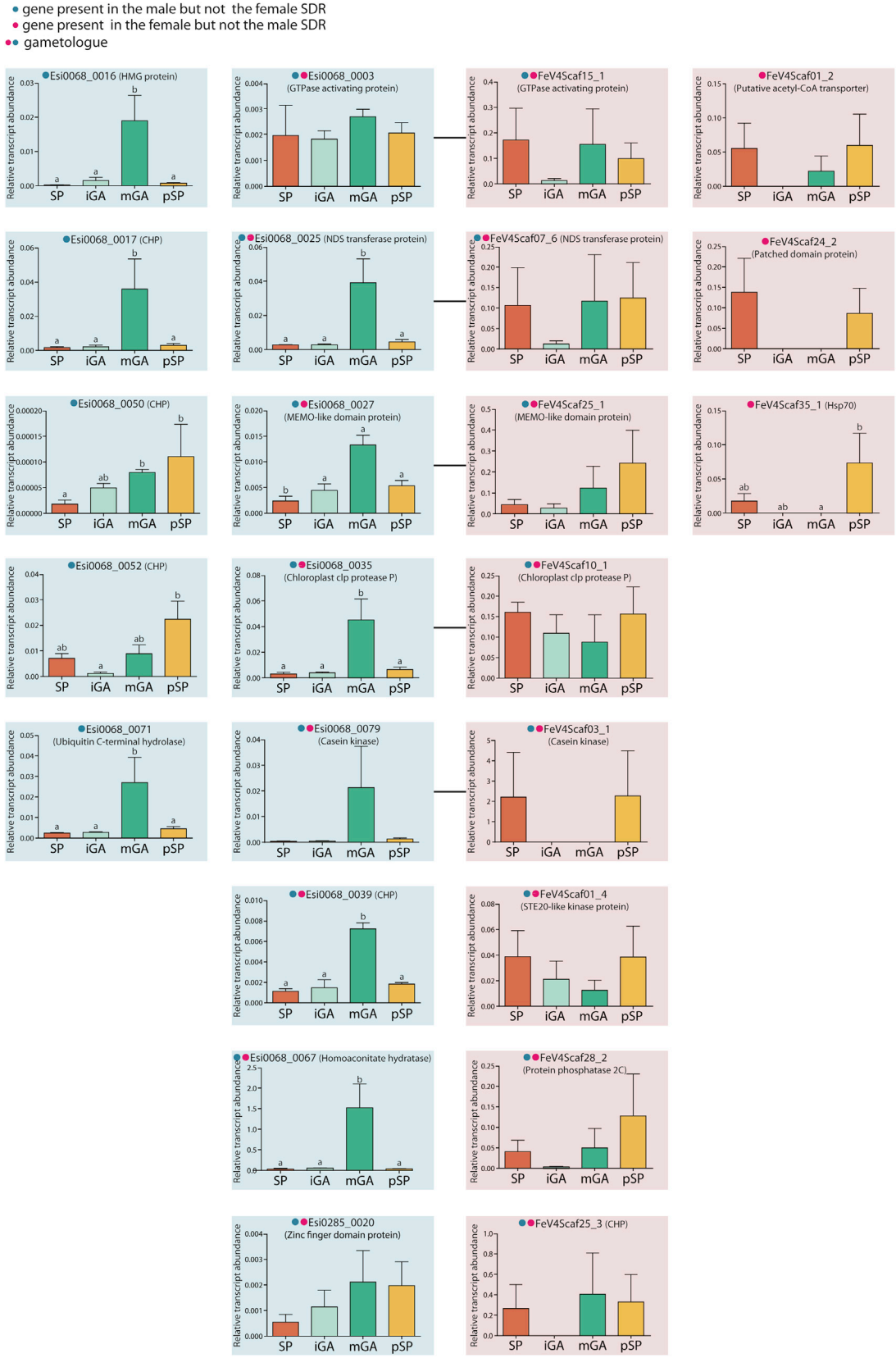

Figure 4. SDR Gene Expression during the Life Cycle

Male and female SDR gene expression during the life cycle of *Ectocarpus* sp. measured by qRT-PCR, relative to a housekeeping gene (EF1α). Gene annotations are indicated in parentheses (see Table S2 for further details). Abundances of transcripts for female and male SDR genes were measured using RNA from

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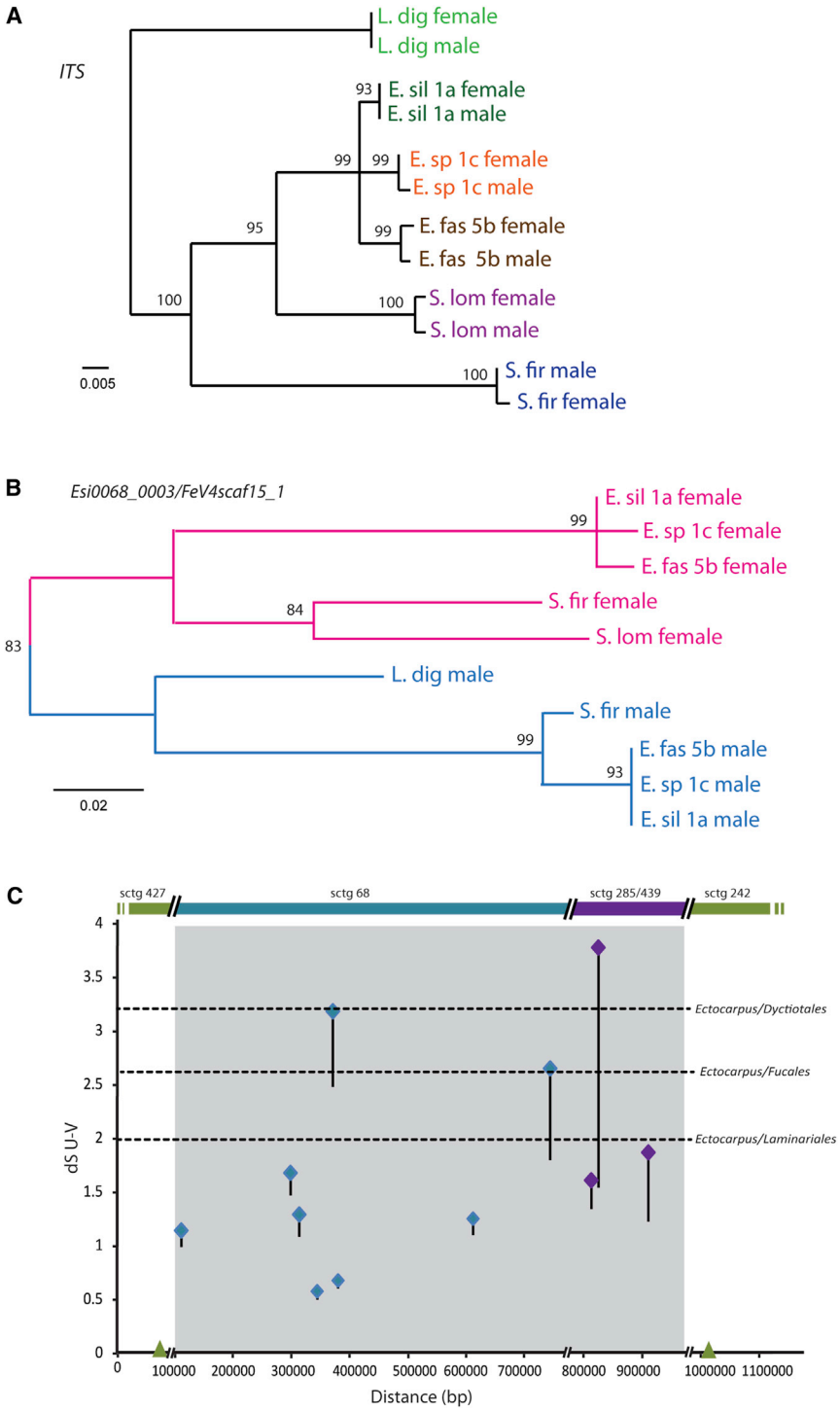

Figure 5. Estimation of the Age of the *Ectocarpus* sp. SDR

(A) Maximum likelihood tree created in MEGA5 [40] based on the Kimura 2-parameter model using sequence data amplified from 453 bases of the autosomal region ITS2 and adjacent 5′-LSU. The percentage of trees in which the associated taxa clustered together (bootstrap values from 1,000 resamplings) is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and by then selecting the topology with the best log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories, +*G*, parameter = 0.2094). Distinct lineages are indicated by different colors. Samples correspond to three different *Ectocarpus* lineages, *E. siliculosus* lineage 1a (E. sil 1a), *E. sp.* lineage 1c (E. sp 1c), and *E. fasciculatus* lineage 5b (E. fas 5b), and three distantly related brown algae, *Sphaerotrichia firma* (S. fir), *Scytosiphon lomentaria* (S. lom), and *Laminaria digitata* (L. dig). Lineage names and sex are indicated at the branch tips. The strains used are described in Table S1A.

(B) Maximum likelihood tree with equivalent parameters to those shown in (A) (gamma distribution, +*G*, parameter = 0.2868) for 148 bases of the sex-linked, exonic region of one gametolog pair (Esi0068_0003/FeV4scaf15_1). Pink and blue indicate sequences from female and male individuals, respectively.

(C) Plot of dS values of gametolog and PAR homologous pairs against gene distance, with gene order according to the male physical map. Blue and purple lozenges represent genes on the two male SDR scaffolds, sctg_68 and sctg_285and439, respectively. Green triangles at each end of the x axis represent two flanking PAR genes. One-sided SE bars represent half the SE of the estimation. Double diagonal bars indicate that the orientation of the locus relative to the flanking PAR is not known. Dotted lines indicate mean levels of synonymous site divergence between *Ectocarpus* sp. autosomal genes and autosomal genes of species from the brown algal groups indicated.

See also Figure S5.

rather than gene loss [8, 9]. The presence of a large proportion of haplotype-specific genes in the *Ectocarpus* sp. SDR, relative to the gametologs, and the expression patterns of many haplotype-specific genes, which indicate a role during fertility, would be consistent with his prediction. However, because there is an autosomal paralog for most of these haplotype-specific genes, it is also possible that functional redundancy of SDR genes and their autosomal paralogs allowed gene loss to occur. Future analysis of additional related SDRs, together with an outgroup species in which the region homologous to the *Ectocarpus* sp.

gametophytes and parthenosporophytes of strains carrying either the U or the V sex chromosome, respectively, and from diploid sporophytes (strains carrying both the U and the V). Bars with different letters are statistically different ($p < 0.05$). Details on the statistical analysis are presented in the Supplemental Experimental Procedures. The colored dots next to gene names indicate whether the gene is a gametolog (blue and pink dots) or whether it is only found in either the male or the female haplotype (blue or pink dot, respectively). Graphs corresponding to gametolog pairs are linked by a horizontal line. SP, diploid heterozygous sporophyte; iGA, immature gametophyte; mGA, mature gametophyte; pSP, parthenosporophyte; CHP, conserved hypothetical protein.

SDR is autosomal, may help to trace changes in SDR gene content over evolutionary time and determine the relative importance of gene gain and gene loss during the emergence of this system.

Despite being ancient, the *Ectocarpus* sp. SDR is quite small. Given the low level of sexual dimorphism in *Ectocarpus* sp. and the small number of genes that show sex-biased expression, both of which suggest that there is limited scope for sexual conflict, the small size of the SDR is consistent with the view that SDR expansion is driven by the evolution of genes with sexually antagonistic effects [1, 52]. In a number of sex chromosome systems, the expansion of the nonrecombining region of the Y (or W) has been shown to have proceeded through several events of recombination suppression, and these recombination events have formed regions with different degrees of X-Y (or Z-W) divergence (evolutionary strata) [4, 53] (reviewed in [1, 49]). The lack of detectable strata is consistent with the conclusion that this region has experienced limited expansion. However, given that strata may be extremely difficult to detect in ancient haploid systems (because both U and V can accumulate rearrangements), we cannot totally rule out the absence of these events. Indeed, recent evidence suggests the possible existence of at least two recombination suppression events in the UV system of the bryophyte *Ceratodon* [14], and therefore that UV systems may acquire evolutionary strata in some cases. Note also that the *Ectocarpus* sp. system provides independent evidence that the age of an SDR does not necessarily correlate perfectly either with its size or with the degree of heteromorphy (e.g., [54, 55]).

In *Ectocarpus* sp., the male SDR haplotype was dominant over the female haplotype, even when three copies of the female haplotype were present. It is therefore possible that femaleness may simply be the default state, adopted when the male haplotype is absent. This situation is comparable to that observed in diverse animal, fungal, and land plant sex-determination systems but differs from that observed with the UV systems of some mosses. In the latter, the male and female factors are codominant, leading to monoicy when both the male and female SDR haplotypes are present in the same gametophyte [56]. Functional differences can therefore be observed between different sex-determination systems, independent of the genetic nature of the system (XY, ZW, or UV).

The male-specific HMG gene is a good candidate for the gene that determines maleness in *Ectocarpus* sp. If this can be confirmed experimentally, it will raise important questions about the evolution of sex and mating-type-determination gene networks across the eukaryote tree, suggesting shared or convergent mechanisms in brown algae, fungi, and animals.

Experimental Procedures

Ectocarpus Culture

Ectocarpus strains were cultured as described [57].

RNA-Seq Transcriptome Data

RNA sequencing (RNA-seq) analysis was carried out to compare the abundances of gene transcripts in male and female mature gametophytes. Synchronous cultures of gametophytes of the near-isogenic male and female lines Ec603 and Ec602 (see Table S1A and Figure S1) were prepared under standard conditions [57] and frozen at maturity. Total RNA was extracted from 2 bulks of 400 male individuals and 2 bulks of 400 female individuals (two biological replicates for each sex) using the QIAGEN Mini kit (http://www.qiagen.com) as previously described [48]. For each replicate, RNAs were quantified, and cDNAs for transcriptome analysis were polythymine primed, fragmented, cloned, and sequenced by Fasteris. We used both de novo assembly (Trinity) (r2012-01-25) [58] and TopHat (v.2.0.8) [59, 60] and Cufflinks (v.2.1.1) [60, 61] algorithms. Statistical testing for sex-biased gene expression was performed using DEseq [62].

Identification and Mapping of the Male SDR

A comparative genome hybridization approach [25] identified several regions of the genome exhibiting polymorphisms between male (Ec32) and female (Ec568) strains. Primers were developed for these putative sex-linked regions, and mapping was performed by genotyping the 60 individuals of the mapping population [26]. Details of the PCR conditions are given in the Supplemental Experimental Procedures. The approaches used to improve the assembly of the male SDR and to verifiy the completeness of the male SDR using both an RNA-seq-based method and an approach based on the YGS method developed by Carvalho and Clark [27] are described in detail in the Supplemental Experimental Procedures.

Recombination Analysis

Recombination between sex locus markers was analyzed using a large segregating family of 2,000 meiotic individuals (Figure S1) derived from a cross between the male line Ec494 [48] and the female outcrossing line Ec568 [26].

Sequencing of a Female Strain and Identification and Assembly of the Female SDR

The genome of the female strain Ec597 (Table S1A and Figure S1A) was sequenced using a whole genome shotgun strategy that involved the implementation of both Illumina HiSeq 2000 technology and Roche 454 pyrosequencing. Velvet (v.1.1.05) was used to run several assemblies during the sequencing process, including the v.3 assembly (which used all the paired-end reads and reads from one of the mate-pair libraries) and the final v.4 assembly with the complete read data set (Table S1E). An independent de novo assembly was also carried out with the CLC assembler (http://www.clcbio.com/products/clc-assembly-cell) using only the paired-end Illumina data.

Female SDR scaffolds were identified using three different approaches. First, we blasted the deduced protein sequences of male SDR genes (all annotated genes on the two male SDR scaffolds sctg_68 and sctg_285and439) against the female genome assembly. Fourteen candidate female SDR scaffolds were identified in the V4 assembly using this approach. Second, we used an approach that employed RNA-seq transcriptome data. Third, we also adapted the YGS method [27] to identify female-linked sequences. These approaches are described in detail in the Supplemental Experimental Procedures. All putative female-specific scaffolds were verified by PCR using between 8 and 57 individuals. Several approaches were used to improve the assembly of the female SDR. Details are given in the Supplemental Experimental Procedures.

Annotation of SDR Scaffolds

The male SDR scaffolds had been annotated as part of the *Ectocarpus* sp. genome project [16], but the gene models were considerably improved by integrating transcript information derived from the RNA-seq analysis carried out as part of this study and by using comparisons of male and female gametolog gene models. The updated gene models can be accessed on the OrcAE database (http://bioinformatics.psb.ugent.be/orcae/overview/Ectsi) [63]. The female SDR scaffolds were annotated de novo by running the gene prediction program EuGène [64], which incorporated the signal prediction program SpliceMachine [65], using the optimized Markov models and SpliceMachine splice site predictions derived previously for the male genome sequence [16]. Gene prediction incorporated extrinsic information from mapping of the RNA-seq data onto the female-specific scaffolds. Both male and female SDR gene models were manually curated using the raw, mapped RNA-seq data, the Cufflinks and Trinity transcript predictions, and the comparisons between the male and female haplotypes.

Pseudogenes were identified manually by comparing SDR sequences with genes in the public databases. An additional screen for pseudogenes was carried out by blasting male protein sequences against the genomic sequence of the female SDR and vice versa. All sequences that had been annotated as "gene" or "TE" were excluded from this latter analysis using Maskseq and RepeatMasker, respectively.

Homologous genes present in both the male and female haplotypes of the SDR were considered to be gametologs if they were detected as matches in a reciprocal BLASTP search against the SDR scaffolds (E value cutoff: 10^{-4}). The same criterion was used to identify homologs of SDR genes located outside the SDR (Table S2).

Identification of Transposons and Other Repeated Sequences in the SDR

An *Ectocarpus*-specific TE library (described in [16]), which had been compiled with REPET [66], was used to annotate SDR transposons. TEs were also annotated by running the de novo annotation software Repclass [67] with default parameters. See the Supplemental Experimental Procedures for details.

Intrahaplotype Sequence Similarity

Analyses of sequence similarity within the male and female SDR haplotypes were performed using a custom Perl code [5]. By default, the threshold for sequence identity was fixed to 97%. When the threshold was reduced to 50%, the same result was obtained.

Quantitative RT-PCR Analysis of SDR Gene Transcript Abundances during the *Ectocarpus* sp. Life Cycle

The abundance of male and female SDR gene transcripts during the *Ectocarpus* sp. life cycle was assessed by quantitative RT-PCR (qRT-PCR). Primer pairs were designed to amplify regions of the 3′UTR or the most 3′ exon of the gene to be analyzed (Table S4D). In silico virtual PCR amplifications were carried out using the electronic PCR program [68] and both the male and female genome sequences to check the specificity of oligonucleotide pairs. qRT-PCR analysis was carried out for 13 male SDR genes and 11 female SDR genes (Figures S4A and S4B). The remaining SDR genes could not be analyzed either because they had very small exons, which posed a problem for primer design, or because it was not possible to obtain a single amplification product. RNA extraction and qRT-PCR were performed as previously described [48].

Construction of Phylogenetic Trees for an SDR and an Autosomal Gene

Exon sequences from an SDR and an autosomal sequence were amplified from three *Ectocarpus* lineages, from *S. firma* (E. Gepp) Zinova and *S. lomentaria* (Lyngbye) Link, distantly related brown alga within the order Ectocarpales, and from the kelp *L. digitata* (Hudson) J.V. Lamouroux. For the SDR gene, an exon region was amplified for the gametolog pair Esi0068_0003 (male) and FeV4scaf15_1 (female). Alignable sequence data from the internal transcribed spacer 2 (ITS2) nuclear autosomal region and adjacent large subunit (LSU) were obtained for the same strains. Sequences were edited using the Codon Code sequence aligner and aligned with Muscle in the program SeaView [69]. Evolutionary history was inferred using both the Neighbor-Joining (Figures 5B and 5C) and PhyML methods implemented in MEGA5 [40], with the same topology resolved by both methods. The strains and lineages used are described in Table S1A, and the primers are described in Table S3.

Synonymous Divergence

Pairwise alignments of the deduced protein sequences of gametolog gene pairs were performed in SeaView using Muscle with default parameters. Regions with poor alignments were further analyzed with Gblocks [70]. The aligned protein sequences were then back translated to coding sequence, and dS was calculated using Codeml within the suite of programs in PAML v.4 [71].

Estimating the Age of the *Ectocarpus* sp. SDR

Coding sequence data from 65 Stramenopile species, including two diatoms, were obtained from the Hogenom database v.6 and from GenBank [72]. Homologous genes were identified using a clustering approach. Orthologous sequences were identified and checked using phylogenetic information (described in the Supplemental Experimental Procedures). Coding sequences from other Phaeophyceae species were added to the cluster data, and further data cleaning was carried out so that only orthologous sequences were retained, as described in the Supplemental Experimental Procedures. A pairwise alignment of the *Ectocarpus* sp. genes with all of the identified orthologous genes from each cluster was then carried out using Prank [73], and alignments were improved using Gblocks [70, 71]. The programs Codeml and Yn00 from PAML v.4 [71] were then run on each gene pair in order to calculate pairwise dS values. The resulting dS values were plotted against the divergence times estimated by Silberfeld et al. [74] and Brown and Sorhannus [75].

Codon Usage Analysis

A set of 27 optimal codons was identified by comparing the codon usage of highly expressed genes (ribosomal genes) with the rest of the genome using the multivariate approach described in Charif et al. [29]. Fop values were correlated with RNA-seq expression levels (Figures S2C and S2D).

Sex Determination in Strains Carrying Different Numbers of U and V Chromosomes

Polyploid gametophytes were constructed using the *ouroboros* mutant [48] (Figure S1A). Details of genetic crosses and ploidy verification are given in the Supplemental Experimental Procedures.

Accession Numbers

The GenBank accession number for the raw sequence data (Supplemental Experimental Procedures) reported in this paper is ERP002539. The SRA accession numbers for the raw sequence data (Supplemental Experimental Procedures) reported in this paper are SRX468696 and SRX468697.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, five figures, and sixteen tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.07.042.

Author Contributions

S.M.C., D.R., J.M.C., and G.A.B.M. designed the research study. S.A., E.P., R.L., A.F.P., S.M.D., and M.R. performed the research study. S.M.C., J.M.C., S.A., A.C., R.L., E.P., G.A.B.M., J.B., L.S., and M.V. analyzed the data. J.-M.A., E.C., and Y.V.d.P. contributed analytic and computational tools. S.M.C. coordinated the research study. S.M.C., S.A., and J.M.C. wrote the manuscript, with input from all the authors.

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III. Discussion and Perspectives

The identification and characterization of the *Ectocarpus* sp. UV sex chromosomes have shown that female- and male-specific non-recombining regions share some homologous genes. Such sex chromosomes homologues are often referred to as gametologues. The presence of these genes strongly suggests that both U and V sex chromosomes have evolved from a pair of autosomes in a manner comparable to that which produced diploid sex chromosome systems, as proposed by Bull in 1978. Several mechanisms have been proposed for the loss of recombination between sex chromosomes in diploid systems: through chromosomal rearrangement or progressive loss of recombination by sexually antagonist selection (Rice, 1987). With regard to the former, chromosomal rearrangements have been shown to be responsible for the evolutionary strata in human and avian sex chromosomes (Bellott *et al*., 2014; Lahn, 1999; Lemaitre *et al*., 2009; Ross *et al*., 2005; Wilson and Makova, 2009; Wright *et al*., 2012, 2014). However, recent analyses have shown that loss of recombination within some sex chromosomes has evolved by gradual events and not by large chromosomal rearrangements (Bergero *et al*., 2013; Natri *et al*., 2013; Qiu *et al*., 2013). In *Ectocarpus* we could not detect evolutionary strata on sex chromosomes. This does not mean that evolutionary strata do not exist in this system as they may be undetectable because the recombination has stopped a long time ago and because both, the U- and the V-specific regions, are evolving rapidly due to loss of recombination (in contrast to X and Y where the X continues to recombine with the X in the females).

To study the molecular events responsible for the cessation of recombination between U and V sex chromosomes, it is important to have a less fragmented assembly of the female sex SDR. In fact, while the male SDR has been sequenced using Sanger technology, is relatively complete and well assembled (Cock *et al*., 2010), the female SDR was sequenced using Illumina methodology, and the lack of BAC libraries or long reads for this highly repeated region precluded the generation of a high quality assembly. Such an improvement in the assembly quality of the female SDR would allow rearrangements within the female and male SDR (*e.g*. duplication, translocation, inversion) to be identified and studied, and to test if the SDR of the UV sex chromosomes lost their recombination capacity due to chromosomal rearrangements or to a mechanism that caused gradual loss of recombination. The Algal

Genetics Group is currently attempting to improve the female SDR assembly using PacBio sequencing technology which produces longer sequence reads (>14 kbp), which will hopefully solve the female SDR assembly problem.

Analysis of expression of sex-linked genes during the life-cycle of *Ectocarpus* indicated that the male-specific gene *Esi0068_0016* is a strong candidate for the male sex-determining gene because it is highly expressed during the male mature gametophytic phase. The gene *Esi0068_0016* codes for a HMG domain protein, a class of protein that is known to be involved in male-sex determination of most mammals (Kashimada and Koopman, 2010) and in gender determination in fungi (Idnurm *et al*., 2008). To further study this HMG-domain gene and its implication in the male-determining pathway, it would be interesting to analyse the function of this gene. Such a study would be feasible using gene silencing techniques, which are currently been developed in the Algal Genetics group. As we know that the male haplotype of the SDR is dominant over the female haplotype, we can use diploid gametophytes that carry both the male and female SDR haplotypes (constructed using the *ouroboros* mutant; Coelho *et al*., 2011) to look for male-to-female transitions following knockdown of each of the candidate male-determining genes using RNAi. Because these diploid gametophytes carry both, the U and the V haplotypes of the SDR, they should become female when the male sex-determining gene is knocked down. In parallel, a TILLING mutant collection, being established in our group, could be screened for mutations in the candidate gene(s) and the sex phenotypes of mutant strains could be determined by crosses with reference female strains. Furthermore, a genetic screen for male-to-female sex-reversed mutants of *Ectocarpus* obtained by mutagenizing diploid UV gametes and visually screening for gamete fusions, is currently in progress.

Measurements of transcript abundance for genes within the male haplotype of the *Ectocarpus* SDR during male gametophyte maturation have identified other genes that are absent from the female SDR and that are highly expressed at this stage of the life cycle. They therefore also represent strong candidates for the sex-determining gene(s). Functions of those genes could be analysed by similar approaches describe above (*i.e*. RNAi and screening for mutations) and therefore test their role in the male-determining pathway.

The discovery of the *Ectocarpus* master sex determining gene(s) and the identification of its downstream targets would have a significant impact on the current understanding of the

evolution of sex-determination gene networks across the Eukaryotes. In particular, if the candidate *Esi0068_0016* can be confirmed as being a master-switch gene for male-determination in *Ectocarpus*, it will suggest either a shared or a convergent mechanism in brown algae, fungi and animals for the determination of sex. However it is important to note that such master effectors in the sex-determining cascade are known not to be conserved, in contrast with the conserved downstream effectors (Graham *et al*., 2003; Graves and Peichel, 2010). In animals, the conserved Doublesex-Mab (DM) genes family have been found to be involved as downstream effectors of the sex-determining cascade of all animals (Miller *et al*., 2003; Raymond *et al*., 1999). This conservation of downstream effectors and the diversity of master effectors have led to the hypothesis that master effectors have independently acquired the master switch gene function because their primary function is suited for triggering the sex-determination cascade (Graves and Peichel, 2010). Therefore, if the *Ectocarpus* HMG-domain triggers the male-determining pathway, this could be the result of an independent acquisition of the gene because its function is particularly suitable for this task.

Analysis of SDR genes and their homology with autosomal genes has revealed some interesting patterns. Seven sex-specific genes are highly similar to (>80% identity) autosomal genes (Figure 3 in Ahmed *et al*., 2014). This similarity could be explained by a duplication of these genes from or to the SDR, occurring after the loss of recombination between U and V sex chromosomes. To determine if these genes have an SDR or an autosomal origin, the ancestral gene content of the SDR needs to be determined. The use of an outgroup would allow such gene movement to be studied and to test if the male and female SDR in *Ectocarpus* evolved by gene gain, as predicted for haploid sex chromosomes systems (Bull, 1978).

While characterising the SDR of the *Ectocarpus* sex chromosomes we noticed that the pseudoautosomal region (PAR) exhibited a number of structural features, such as gene density and TE density, that were intermediate between those of the SDR and autosomes. This was surprising because genetic mapping (Heesch *et al*., 2010) indicated that the PARs recombine during meiosis. In order to address this issue, we performed a detailed investigation of the recombining portions of the *Ectocarpus* sex chromosome. This analysis is described in the next chapter.

Chapter 3. The Pseudoautosomal Region on the *Ectocarpus* UV Sex Chromosomes

I. Introduction

Sex chromosomes often maintain regions of sequence homology where recombination persists. These regions are known as pseudoautosomal regions (PARs). Because recombination is maintained, PARs are expected to resemble autosomes but their close proximity to the non-recombining region of the sex chromosomes is expected to have important consequences for their patterns of inheritance and consequently for their evolutionary fate. The relative time that each sex chromosome spends in each sex determines to what degree a sex chromosome is under female or male-specific selection. For instance, in XY systems, the Y chromosome is only inherited by sons and X chromosomes spend two-thirds of their time in daughters. Models predict that this difference of time spent in each sex mediates the accumulation of female-beneficial alleles on X chromosomes and male-beneficial alleles on Y chromosomes (Charlesworth *et al*., 1987; Rice, 1984). Therefore PARs are expected to maintain sex-specific polymorphisms, which make them regions where accumulation of SA genes is expected, particularly for regions in the vicinity of the non-recombining region (Charlesworth *et al*., 2014; Otto *et al*., 2011). This pattern of sexual antagonistic (SA) allele distribution with respect to the SDR is expected to favour the loss of recombination and expansion of the non-recombining region in a stepwise fashion (see Chapter 1). However in UV sex chromosomes system, no models have been proposed so far to predict how PARs should evolve and there is no theoretical prediction for the accumulation (or not) of SA alleles.

Progressive expansion of the SDR over evolutionary time through acquisition of SA alleles would suggest that at some point, the sex chromosomes would become entirely non-recombining. However, the absence of PARs is rare, suggesting that there are selective pressures to prevent complete loss of recombination and to maintain those regions. In mammals, the maintenance of PARs is crucial for the preservation of correct segregation of the sex chromosomes and this is thought to be the main explanation for the persistence of PARs in these organisms (Rouyer *et al*., 1986; Shi *et al*., 2001; Soriano *et al*., 1987). However, in some rare cases sex chromosomes lack PARs and are still able to segregate, for example in marsupials (Patel *et al*., 2010) or in *C*. *elegans*, where there is no Y chromosome

to pair with (Madl and Herman, 1979). These examples suggest that there may be additional reasons for the maintenance of PARs. Indeed other forces can act to favour the maintenance of recombination on PARs and counteract sexually antagonistic forces: Hill-Robertson effects can favour positive genetic association through recombination and the prevention of Muller's ratchet effects (see Box 2 in Chapter 1; Barton & Charlesworth, 1998; Otto, 2009). There may also be mechanical explanations for the maintenance of the PAR, for example in *Silene latifolia* where there is evidence of translocation of genetic material into the PAR. Bergero *et al*. (2013) have shown that the *S*. *vulgaris* autosome that corresponds to the X chromosome in *S*. *latifolia*, does not share any genes with the *S*. *latifolia* PAR, which suggest that the PAR was added to the *S*. *latifolia* sex chromosomes at a later stage. This type of translocation can be favoured if it carries genes that are under SA selection allowing close genetic linkage between the SDR and sexually antagonistic genes (Lenormand, 2003; Otto *et al*., 2011).

The specific evolutionary dynamics of the PAR remain poorly understood (Otto *et al*., 2011). We know very little about general gene content and structure of PARs, and the information we have comes mainly from animals, which have relatively small PARs (with the exception in plant with *Silene* PAR). Also, theoretical models lack for UV systems. The sequencing of the *Ectocarpus* sex chromosomes (see Chapter 2) together with the fact that a large proportion of those UV chromosomes are recombining, offers an opportunity to look at the structure of the PAR in a UV system. Our recent work, described in Chapter 2 has shown that the *Ectocarpus* UV sex chromosomes are extremely ancient, having evolved at least 70 million years ago (Mya), and probably more than 100 Mya (Ahmed *et al*., 2014). Despite their age, the non–recombining, sex-determining region is relatively small, compared with sex chromosomes of similar age in other systems. The SDR occupies about a fifth of the sex chromosome, and is surrounded by two relatively large PARs.

We used an experimental and modelling approach to characterize the PARs of *Ectocarpus*. We show that recombination events are unevenly distributed along the PARs, and reveal the distinct evolutionary features of this region. These include accumulation and accelerated evolution not only of sex-biased genes but, remarkably, of genes differentially expressed during the gametophyte versus sporophyte generation of the life cycle (generation-biased genes). In agreement with the experimental data, our theoretical model predicts that the evolution of the PAR in haploid sex-determining systems may be shaped by generation-antagonism, provided that different selection pressures act on males and females.

II. Paper

Title:

The unique features of the pseudoautosomal region of the U/V sex chromosome system in the brown alga *Ectocarpus*

(Article in preparation)

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R.L. contributed to this paper by analyzing the recombination rate, structural characteristics, the evolutionary rates of PAR genes and drafting the manuscript.

Abstract

The pseudoautosomal regions (PARs) of sex chromosomes are unique genomic regions. However, they are notorious difficult to study, and consequently very few PARs have been characterised. Here, we describe the genomic and evolutionary features of the extensive pseudoautosomal regions (PARs) that border the sex determining region on the *Ectocarpus* U/V sex chromosome. We show that recombination events are unevenly distributed along the PARs, with recombination hotspots bordering the sex-determining region, and we reveal the distinctive structural features that make PARs intermediate to autosomal and sex-linked, non-recombining sequences in several genomic and evolutionary respects. Remarkably, we find that the *Ectocarpus* PAR is enriched in genes whose expression is restricted to the sporophyte generation (generation-biased genes), and propose a theoretical model to explain this observation. This study represents the first genetic and evolutionary analysis of PARs in a UV sexual system and demonstrates that despite their extent relative to the SDR, PARs of undifferentiated UV sex chromosomes exhibit unique characteristics across their whole length.

Introduction

Sex chromosomes often display strikingly distinctive features, including differences in size, structure and gene content, and more marked levels of sex-biased gene expression compared with autosomes. These characteristics are thought to result from the suppression of recombination between the sex chromosomes. A broadly established model of sex chromosome evolution implies gradual expansion of recombination restriction between the X (or Z), the Y (or W) and the U and V chromosomes, driven by selection for linkage between the sex-determining region and loci at which selection differs between males and females (1, 2). This is accompanied by the concomitant contraction of the so called pseudoautosomal region (PAR). Despite the evolutionary tendency to reduce recombination across the whole length of the sex chromosome, most species retain sequence homology in the PAR, and this is thought to be because homologous recombination in this region plays a critical role in chromosomal pairing and segregation during meiosis (3, 4). Moreover, there are situations where sexually antagonistic forces may be too weak to drive the expansion of the SDR, and a relatively extensive PAR may be preserved, for instance in organisms where the phenotypic

differences between male and females are very low (e.g. (5)). Likewise, large PARs can be maintained if other forces such as restriction of gene expression to the right sex (sex-biased gene expression) resolve sexual antagonism (6) if dosage compensation is lacking (7) or through occasional X-Y recombination, which can eliminate accumulated deleterious alleles (8).

Interest in the evolutionary dynamics of the PAR has increased over recent years. The evolutionary fate of PAR genes is expected to differ from either autosomal or fully sex-linked genes. In particular, sex differences in allele frequencies should be maintained more easily in the PAR, either due to sexually antagonistic polymorphisms (which are maintained under wider ranges of conditions than on autosomes), or to polymorphisms retained by other forms of selection, such as heterozygote advantage (9). How far should this effect extend along the PAR depends on the strength of selection maintaining polymorphism, relative to the rate of recombination between the selected locus and the SDR (10, 11). However, despite the increasing amount of theoretical predictions made for the PAR, empirical work on this intriguing genomic region is still in its infancy. Genomic data on the character and structure of the PAR mainly come from organisms which have old and differentiated sex chromosomes such as humans and other mammals (12, 13), and more recently birds (14). These PARs have been shown to exhibit increased recombination levels and several distinct structural characteristics compared with autosomes, such as different density of repetitive DNA (14, 15), and distinct GC percentage and gene evolutionary rates that were intermediate between the sex-linked and autosomal regions (14).

All studies mentioned above have focused on PARs of diploid sex chromosome systems (XY and ZW). However, in a large number of taxa, in particular algae, fungal, plant taxa, sex is determined during the haploid phase of the life cycle. The notation UV has been proposed for such haploid systems (where U and V stand for the female and male chromosome, respectively) in order to distinguish them from diploid XY and ZW systems (16). The evolution of recombination suppression and the maintenance of sex differences in allele frequencies in the PAR are also expected in haploid systems, as recently shown by (2). However, empirical data on the genomic structure and the evolutionary features of PARs in organisms with haploid sex determination are currently lacking.

We have recently shown that the 70 MY old UV sex chromosomes of the brown alga *Ectocarpus* present a surprisingly small non-recombining, sex-determining region (SDR) that is bordered by two relatively large PARs (5). Here, we used experimental and modelling

approaches to carry out a detailed analysis of the *Ectocarpus* PARs. We present a recombination mapping of this region, analyse its gene content and expression during the haploid-diploid life cycle of *Ectocarpus* and reveal the evolutionary features of this remarkable region.

RESULTS

The pseudoautosomal region of the *Ectocarpus* sex chromosome exhibits unusual structural features

The PAR region of the *Ectocarpus* sex chromosome (linkage group 30, LG30) represents about 2 Mbp of sequence on each side of the 1 Mbp SDR. We have previously noted that the PAR exhibits a number of structural differences compared to the autosomes. For instance, values for gene density, mean intron length, and percent GC content are intermediate between those of the autosomes and the SDR (5).

It has been shown in avian and mammalian species that different size classes of chromosomes exhibit a number of correlated attributes. In birds, for instance, micro-chromosomes (<20Mbp) have different properties in terms of GC content, repeat content, gene density, intron size and recombination rate compared with larger chromosomes (17, 18). Similarly, in rat, mouse and human the size of a chromosome is correlated not only with recombination rates but also with GC content and number of transposable elements (19). Taken together, these studies suggest that chromosome size should be taken into account when comparative analyses of chromosome structure are carried out. Consequently, to analyse in detail the unusual structural features of the *Ectocarpus* PAR, we compared the sex chromosome to linkage group 4 (LG04), an autosomal linkage group of similar size. For this comparison, all genes on LG30 and LG04 were manually curated to produce high quality annotations for both chromosomes. Comparison of these two genomic regions confirmed that both GC content and gene density were significantly lower for PARs, compared to the LG04, and that PAR genes tended to have longer introns, on average, than genes on LG04 (Fig. 1A). Moreover, the PARs contained more transposable element sequences and the PAR genes both had fewer and smaller exons on average than genes on LG04 (Fig. 1B-H). All of these differences were also detected at a significant level when the PARs were compared with the total autosomal region of the genome (all chromosomes apart from the sex chromosome). These analyses therefore confirmed that the PARs exhibit a number of unusual features compared to the autosomes.

The PAR exhibited some structural heterogeneity along its length, with for example a significant negative correlation between TE content and gene content (Pearson's correlation test, $p<0.01$), but we found no evidence that the features that distinguish the PARs from the autosomes (gene structure, GC content, etc.) were more marked in the vicinity of the SDR (Table S1). These unusual structural features are therefore characteristic of the entire PAR.

Recombination along the sex chromosome

The structural analysis described above strongly indicated that the *Ectocarpus* PARs exhibits features resembling those of the non-recombining SDR. Recombination is totally suppressed within the SDR of the *Ectocarpus* sex chromosome (5) but analysis of molecular marker segregation has shown that the PARs recombine during meiosis (20). In order to study in more detail the recombination rates across the PAR, an additional 23 markers and a total of 280 individuals were used to build a more comprehensive recombination map of the *Ectocarpus* sex chromosome. The average recombination rate in the PAR (320 cM/Mb; excluding the SDR) was not significantly different from the genome average (230 cM/Mb; Mann-Whitney U-test, p-value=0.28) but recombination events were unevenly distributed along the sex chromosome (Fig. 2). Specifically, two regions of high recombination (one of them recombining at about ten times the genome average) were found on each side of the SDR. When these hotspots were excluded from calculations, the PAR had an average recombination rate of 140 cM/Mb, which was still not significantly different from the genome average. Thus, based on these segregation analyses in the SDR flanking regions, we conclude that the recombination frequency on both sides of the SDR is significantly higher than the genome average, implying the presence of a mechanism that enhances recombination in this region of the genome. Globally, we found no significant correlation between recombination rate and TE or gene content (Pearson correlation tests, $p >0.05$) along the PAR sequence, although there was a tendency for regions that exhibited higher recombination rates to have higher gene density and lower TE density (Fig. 2).

Genetic recombination rates along the PAR was also studied in a segregating family generated from two parental strains of another lineage of *Ectocarpus*, *E. siliculosus* lineage 1a, confirming that recombination events do take place in the PAR of this sister species. (Fig S1).

Expression patterns of PAR genes during the *Ectocarpus* life cycle

The PAR region contains 250 protein coding genes. We investigated the patterns of gene expression of the PAR genes at several stages of the haploid-diploid life cycle of *Ectocarpus* using RNA-seq. The life cycle stages included male and female immature and fertile gametophytes, and also different tissues of the sporophyte generation. The PAR genes exhibited significantly lower mean expression levels than genes in LG04 (Wilcoxon test, $P=4.50E^{-10}$) (Fig. 3A,). A similar trend was observed when the PAR was compared with all the autosomes (Wilcoxon test, $P<1.10E^{-07}$). This difference in transcript abundance was particularly marked during the gametophyte generation of the life cycle, and slightly less significant during the sporophyte generation.

A heatmap based on the RNA-seq data reflecting the expression level of each PAR gene in relation to its position on LG30 (excluding the SDR) revealed a striking pattern (Fig. 3B, Fig. S3A). Several physically-linked clusters of genes that exhibiting similar expression patterns during the life cycle were detected, including two clusters of PAR genes that were strongly up-regulated during the sporophyte-generation, and a cluster of genes that exhibited very low levels of transcription, below the detection limit (RPKM<1), during both gametophyte and sporophyte generations of the life cycle.

To further analyse the relationship between genomic location and life cycle expression pattern, we carried out a genome-wide analysis to identify genes that were differentially expressed during the alternation between the sporophyte and gametophyte generations of the life cycle. About 17% of the genes in the *Ectocarpus* genome was found to be significantly differentially regulated between the generations (FC>=2, FDR<0.1), with sporophyte-biased genes constituting about 9% of the genome (1,484 genes) and gametophyte-specific genes about 8% (1,288 genes). Statistical analysis indicated that the PAR is a preferential location for generation-biased genes, in particular genes that are up-regulated during the sporophyte generation (chi-square test, $p_{adj} = 6.03E^{-05}$, Bonferroni correction) (Fig. 3C). This feature was specific to the sex chromosome, since none of the autosomes exhibited a significant enrichment in sporophyte-biased genes (Fig. S4).

To examine the relationship between level of expression and degree of generation-bias, the sporophyte-biased genes on the PAR and on LG04 were grouped according to fold-change in transcript abundance between the sporophyte and gametophyte generations, and the mean expression level (RPKM) of each group was plotted (Fig. 3D). For LG04, these plots indicate that, when genes exhibited high levels of sporophyte-biased expression (high fold change) this was because they exhibited lower levels of expression in the gametophyte generation

compared with the genes that exhibited lower levels of sporophyte-biased expression (lower fold change). In other words, for this chromosome the degree of sporophyte-biased expression was determined by the level of expression in the gametophyte. In strike contrast, all the sporophyte-biased genes on the PAR exhibited very low levels of expression in the gametophyte-generation (below the detection threshold, RPKM<1) and the degree of sporophyte-biased expression (fold change) was determined by the level of expression during the sporophyte-generation. Hence, sporophyte-biased expression of PAR genes appeared to be principally a result of the genes being silenced during the gametophyte generation.

Forty-three sporophyte-biased and 16 gametophyte-biased genes were identified on the PAR. A significant proportion (ca. 50%) of the sporophyte-biased genes on the PAR were located in the two life cycle gene clusters mentioned above. In these clusters, eight out of nine (sctg_266) and 13 out of 19 (sctg_96) contiguous genes exhibited sporophyte-specific expression (Fig. S3A). Clustering analysis confirmed that the distribution of sporophyte-genes on the PAR was not random (Runs test, $P=2.39E^{-7}$). The sporophyte-biased genes in the two clusters included a duplicated pair of adjacent genes for which there was one copy in each cluster (Table S2). The regions corresponding to the clusters did not exhibit unusual patterns of recombination compared to the rest of the PAR. The remaining sporophyte-biased genes were distributed along the PAR in triplets (1), pairs (4) or individually (11) (Fig. S3A). Neither functional domains nor orthologues in public databases were detected for the majority of these genes (and indeed for the majority of PAR genes in general) and it was not therefore possible to identify any enrichment with respect to function. However, possible roles in protein-protein interactions (leucine rich repeats, tetratricopeptide repeats or ankyrin repeats motifs) were predicted for 7 of the 43 sporophyte-biased PAR genes. The generation-biased genes on the PAR displayed no unusual structural characteristics compared with unbiased PAR genes (Fig. S3B).

A small proportion of the genes in the *Ectocarpus* genome exhibits sex-biased gene expression (5), including 31 that are located in the PAR. This latter set of genes did not display any unusual structural characteristics compared with unbiased PAR genes (Fig. S3B). There was also no significant tendency for generation-biased genes to be also sex-biased (chi-square test, p-value = 0.25). Nonetheless, 12 of the 59 generation-biased on the PAR exhibited both generation- and sex-bias and there was a marked correlation between the precise type of life cycle generation-bias and the type of sex-bias: all seven of the genes that were both gametophyte-biased and sex-biased were male-biased, whereas four out of five of

the genes that were both sporophyte-biased genes and sex-biased were female-biased (Table S2).

Evolution of the PAR genes

The rate and pattern of evolution of *Ectocarpus* genes was analysed by comparing sequences from the reference strain (*Ectocarpus* sp. lineage 1c) with orthologous sequences from another *Ectocarpus* species (*Ectocarpus siliculosus* lineage 1a). Compared with a set of 48 randomly selected autosomal genes from LG04, the 96 PAR genes analysed displayed, on average, significantly elevated values for non-synonymous to synonymous substitution ratios (dN/dS) (Wilcoxon test $p<0.001$). However, when sporophyte-biased genes (39 genes) were removed from the PAR gene set, no significant difference in mean dN/dS ratios was detected between the PAR and autosomal gene sets. Moreover, the sporophyte-biased PAR genes showed dN/dS ratios that were significantly higher than sporophyte-biased genes on LG04 (Wilcoxon test, $p = 2.268e^{-05}$) (Fig. 4A), indicating that the increased evolutionary rates were related to the fact that these generation-biased genes were located on the PAR. The faster rate of evolution of the sporophyte-biased PAR genes was due to an increase in the rate of non-synonymous substitutions (dN) and not to a decrease in the rate of synonymous substitution (dS) (Fig. 4B,C) (Kruskal-Wallis test, $p<0.01$). Finally, note that although the average dN/dS ration for unbiased PAR genes was similar to that of the autosomal gene set, the average values for both dN and dS were significantly greater than for the autosomal genes (Kruskal-Wallis test, $p<0.05$).

Of the 39 sporophyte-biased PAR genes analysed, 24 had dN/dS ratios that were greater than 0.5, which could be an indication of adaptive evolution (21). To perform a maximum likelihood analysis of positive selection (PAML), we searched for orthologues of the sporophyte-biased genes using transcriptome data for two additional *Ectocarpus* species (*E. fasciculatus* lineage 5b and *Ectocarpus* sp lineage 1c Greenland). Complete sets of four orthologous from the four species were obtained for only seven of the sporophyte-biased PAR genes and the PAML analysis was therefore carried out using these sets. Reasons for the low amount of orthologs found is both the limited amount of genomic data for the brown algae but also the fact that PARs may present substantial evolutionary divergence in structure and sequence between species (White et al 2012). For one of these comparisons both pairs of models (M1a-M2a, M7-M8) suggested positive selection (Esi0096_0082, ω=0.86, $p<0.05$).

Codon-usage bias has been observed in almost all genomes and is thought to result from selection for efficient and accurate translation of highly expressed genes (22). Optimal codons have been recently described for *Ectocarpus* (5, 22) and a weak but significant correlation was noted between codon usage bias and gene expression levels (23). Although the genes on the PAR were expressed at a lower level, on average, than LG04 genes (Fig. 3A; Wilcoxon test, $p = 0.0004$), there was no significant difference in the frequency of optimal codons (CAI) compared with the genes on LG04 (Wilcoxon test, $p=0.318$) (Fig. S5A). However, when this analysis was carried out using only the sporophyte-biased PAR genes, the codon adaptation indexes were significantly lower than for LG04 genes (Kruskal-Wallis with Dunn's post-test, $p<0.001$) (Fig S5B). This could be possibly because the sporophyte-biased genes have narrower window of expression (they are silenced during the gametophyte phase), as broadly expressed genes usually have a higher level of bias than do tissue-specific genes (Urrutia and Hurst 2001).

Breadth of expression of PAR genes

Two types of measurement can be used to describe the expression of a gene in a multicellular organism: the level of gene expression in terms of the number of transcripts present in a particular tissue, and the breadth of expression, which relates to how often the gene is expressed through the life cycle and/or in how many different tissues it is transcribed. Breadth of expression can be expressed in terms of the specificity index (see Materials and Methods) where a high specificity index indicates a greater tendency to be expressed specifically in a limited type of tissue and/or at particular stages of the life cycle.

The breadth of expression of *Ectocarpus* genes was calculated using gene expression data collected for multiple tissues and at different stages of the life cycle, and represented using the specificity index (τ). Gametophyte- and sporophyte-biased PAR genes had τ values that were significantly higher than those of unbiased PAR genes or autosomal (LG04) genes (Fig. S6A,B). In contrast, no difference in breadth of expression was detected when we compared 1) gametophyte-biased PAR genes with sporophyte-biased PAR genes, 2) generation-specific PAR genes with generation-specific autosomal (LG04) genes or 3) the sets of PAR and autosomal genes that showed no generation-biased expression.

A model for the evolution of generation-biased genes in the PAR

In XY or ZW systems, it has been argued that the excess of sex-biased genes often observed on X (or Z) chromosomes may result from sexually-antagonistic selection (e.g., (24)). For example in XY systems, alleles with recessive or partially recessive effects that increase male fitness at a cost to female fitness are expected to spread more easily on the X than on autosomes; in a second step, modifiers that decrease the expression of these genes in females may spread, leading to an excess of male-biased genes on the X. We used a theoretical model to explore whether a similar scenario (involving generation-antagonistic rather than sexually-antagonistic selection) could possibly explain the excess of sporophyte-biased genes observed on the PAR. This would imply that alleles increasing the fitness of sporophytes at a fitness cost to gametophytes spread more easily in the PAR than on autosomes, and subsequently trigger the evolution of reduced gene expression in gametophytes.

Our model (detailed in the Supplementary Material) is similar to the model analyzed by (2) and considers a selected locus located at a recombination distance r from the SDR, at which two alleles may have different effects on the fitness of sporophytes, female gametophytes and male gametophytes. However, while (2) explored conditions under selection favours decreased recombination between this locus and the SDR, we focus on the conditions for the spread of a rare allele (say allele a) at the selected locus, as a function of r, the fitness effect of the allele on sporophytes (s_d), female (s_f) and male (s_m) gametophytes. We focus on generation-antagonistic alleles (s_d and $s_h = (s_f + s_m)/2$ have opposite signs), since the spread of such alleles may trigger the evolution of differences in gene expression between sporophytes and gametophytes.

Overall, our analysis (explained in the Supplementary Material, and illustrated in Figure 5) shows that genomic localisation has little effect on the spread of alleles when selection is similar in both sexes ($s_f \approx s_m$); however, when selection differs among sexes (and in particular when the gametophyte-deleterious allele is neutral or slightly beneficial in one of the sexes), linkage to the SDR may greatly benefit to the sporophyte-beneficial allele, which may then avoid being in the sex where it is disfavoured. Linkage to the SDR also benefits to the gametophyte-beneficial allele but to a lesser extent, since this allele still pays a fitness cost in the sporophytic generation. Therefore, taking into account the possibility of sex differences in selection, being in the PAR benefits more to alleles that increase the fitness of sporophytes, at a cost to gametophytes (on average). This model could thus explain the observed excess of sporophyte-biased gene expression in the PAR, under the assumption that reduction in expression in gametophytes evolved secondarily to prevent the expression of alleles that are

deleterious in at least one sex (note that complete linkage to the SDR corresponds to another possible resolution of this conflict).

Discussion

The *Ectocarpus* PAR does not exhibit an increased recombination rate but does have recombination hotspots

PARs play a critical role in the successful progression through meiosis in the heterogametic sex of most plant and animal species because at least one crossover is required for correct segregation of the sex chromosomes (e.g. (25, 26)) generating a strong selective force to maintain recombination in the PAR. Accordingly, in human males, PAR1 has a crossover rate that is 17-fold greater than the genome-wide average. In contrast, the recombination rate in females, where recombination is between homologous X chromosomes, is comparable to the genome-wide average (13, 27). In UV systems, meiosis occurs in the sporophyte and, consequently, there is no male or female meiosis and all meiotic events involve pairs of U and V chromosomes in which recombination can only occur between the PAR regions. This feature of UV systems might be expected to further increase recombination rates in the PAR, but measurement of the recombination rate along the *Ectocarpus* PAR indicated a mean rate that was not significantly different from that of the rest of the genome. The absence of a detectable increase in recombination rate is probably explained by the large relative size of the PAR in *Ectocarpus*. Despite its ancient origin (at least 70 million years ago), the *Ectocarpus* sex chromosome has maintained a large PAR region and a relatively small SDR, the latter being restricted to approximately a fifth of the chromosome (5). Approximately 80% of the chromosome is therefore able to recombine in UV chromosome pairs. Note that the structure of the *Ectocarpus* sex chromosome is consistent with the observation that the age of a sex chromosome is not necessarily correlated with the size of its recombining region (9, 28).

Although the mean recombination rate along the PAR was comparable to that measured for autosomes, recombination mapping identified two hotspots with elevated recombination rates flanking the SDR. Recombination hotspots at borders of SDRs have been described for species with XY or ZW sexual systems, including humans (13), mice (29),blood flukes (30) medaka fish (31), flycatcher birds (14) and papaya (26). A similar phenomenon has also been observed in fungal mating type chromosomes (31). Increased recombination levels in regions flanking the *Ectocarpus* SDR may serve to prevent the non-recombining region from

expanding and eventually capturing the entire chromosome, as it has been suggested for fungal mating type chromosomes (31).

The PAR exhibits structural characteristics that are typically observed in non-recombining regions of the genome

A number of structural features of the *Ectocarpus* PAR region, including TE and gene density and gene structure parameters such as exon size, intron size and GC content of the CDS, were intermediate between the values measured for autosomes and for the non-recombining SDR. Moreover, PAR genes were also expressed at lower levels, on average, than autosomal genes and comparisons with orthologues in other *Ectocarpus* species indicated higher rates of both synonymous and non-synonymous substitutions (and higher dN/dS ratios) in the PAR genes compared with autosomal genes. All of these features are typical of genomic regions that exhibit reduced levels of genetic recombination (5) but, paradoxically, the mean recombination rate measured for the PAR was not significantly different from that of the autosomal part of the genome. Moreover, we found no evidence that PAR genes, on the whole, contained higher levels of sub-optimal codons than autosomal genes (but note that PAR gene coding regions are significantly shorter than those of autosomal genes and this might counteract any tendency for sub-optimal codons to accumulate, because selective pressures on codon usage are typically stronger for genes that encode short proteins (32)).

We considered possible evolutionary mechanisms that might explain the unusual structural and functional features of the PAR and its constituent genes. Genetic linkage to the SDR is expected to influence the evolution of the PAR, but the effect should be limited to regions of the PAR that are very close to the SDR (11). This was not the case for the *Ectocarpus* PAR, as the unusual structural features were characteristic of the entire PAR and were not limited to regions adjacent to the SDR. To date, no mechanisms have been proposed which would allow the SDR to influence the evolution of linked, recombining regions over the distances observed here. It is not clear at present, therefore, whether the unusual structural features of the *Ectocarpus* PAR are related in some way to the presence of the SDR on the same chromosome or if they indicate that the evolutionary history of the PAR has been different from that of the other autosomes. Similar features, in particular enrichment in TEs, have been observed for the human PAR1, which is of similar size (2.7 Mbp) to the two pseudoautosomal regions in *Ectocarpus* but associated with a much larger SDR (15, 33), but it has not been reported whether this phenomenon was limited to the part of the PAR that was adjacent to the

SDR. To further explore the unusual features of the *Ectocarpus* PAR, it will be of interest to determine whether this region undergoes recombination in other brown algal species.

Preferential accumulation of sporophyte-biased genes on the PAR

The *Ectocarpus* PAR is enriched in sporophyte-biased genes compared with the autosomes and these sporophyte-biased genes appear to be evolving in a different manner to the other genes on the PAR. PAR genes in general showed elevated levels of both synonymous and non-synonymous mutations compared to autosomal genes whereas the sporophyte-biased PAR genes showed highly elevated rates of non-synonymous mutations but a similar synonymous mutation rate to autosomal genes. The elevated rate of non-synonymous mutation could be indicative of adaptive evolution, and indeed a signature of positive selection was detected for one out of the seven sporophyte-biased PAR genes that could be analysed for this feature. However, whilst positive selection may be driving the evolution of some of the sporophyte-biased genes, this is unlikely to be the case for all of them. The set of sporophyte-biased PAR genes had a reduced content of optimal codons compared to an autosomal gene set, suggesting that they are under relaxed purifying selection. One possible explanation for the accumulation of non-optimal codons in these genes is that they may escape haploid purifying selection (34) (35) (36). In contrast to autosomal sporophyte-biased genes, which tend to show at least a low level of expression during the gametophyte generation, the sporophyte-biased genes on the *Ectocarpus* PAR are completely silenced during the gametophyte generation. Consequently, alleles with sub-optimal codons will be masked in diploid heterozygous individuals and will not be selected against during the haploid phase.

Another possibility is that the lack of expression of the sporophyte-biased PAR genes during the gametophyte generation leads to relaxed selection by reducing the breadth of expression of these genes. Breadth of expression, i.e. the degree of tissue or developmental stage specificity, is known to effect non-synonymous substitution rates (37). However, this hypothesis alone is not sufficient to explain the higher evolutionary rates of sporophyte-biased genes, because gametophyte-biased PAR genes, which also have a reduced breath of expression, had similar non-synonymous mutation rates to an average PAR gene.

Mathematical modelling was used to identify evolutionary mechanisms that might explain the preferential accumulation of sporophyte-biased genes in the PAR. The model presented here predicts that ploidally-antagonistic genes will spread preferentially in a sexual population if

different selection pressures act on the genes in males and females and if they are genetically linked to the SDR. This model may explain our empirical observations that generation-biased genes accumulate preferentially on the PAR, provided that differences in expression between generations result from generation-antagonistic selection. Our scenario would therefore involve both generation-antagonistic and sex-antagonistic selection, and also implies suppression of expression in both sexes of alleles that are deleterious in one sex only. A similar model recently proposed by (2) similarly predicted that linkage to the SDR would be favourable for loci that are subject to balancing selection (including overdominance and ploidally antagonistic selection) with alleles that are subject to different selection pressures in males and females.

Sporophyte-biased genes in the PAR occur in clusters

Almost half of the sporophyte-biased PAR genes are located in two gene clusters that are highly enriched in sporophyte-biased genes. At present it is not clear why these genes have formed clusters on the PAR. The model presented in this manuscript predicts the accumulation of sporophyte-biased genes near the SDR and could lead to clustering. However, neither cluster is adjacent to the SDR, although it is possible that the clusters have translocated to their current positions as a result of sex chromosome rearrangements. Gene duplication has not played a major role in the evolution of these clusters although there are paralogous pairs of two genes across the two clusters. Clustering of genes with related functions does occur in eukaryotic genomes, although to a lesser extent than in prokaryotes (38, 39), but the *Ectocarpus* genome as a whole does not exhibit unusually high levels of functional clustering (40).

METHODS

***Ectocarpus* culture**

Ectocarpus strains were cultured as described (41).

Fine recombination map

A segregating population of 60 individuals that had been used for the genetic map (42) and additional 220 individuals from a segregating population derived from a cross between strains Ec494 (male) and Ec568 (female) (5) were used to quantify recombination more finely across the pseudoautosomal region. Simple sequence repeat (SSR) markers were already available

for each of the 20 supercontigs of the PAR region of the sex chromosome (LG30). Polymerase chain reactions (PCRs) for SSR genotyping were prepared in 5µL final volumes containing 1.59 µL of sterile Millipore water, 1µL of 10% skimmed milk, 1µL of 5x GoTaq reaction buffer, 0.25µL of DMSO, 10 nmol of $MgCl_2$, 0.5 nmol of each dNTP, 0.2 pmol of the forward primer (which included a 19 nucleotide tail corresponding to a sequence of the M13 bacteriophage), 2 pmol of the reverse primer, 1.8 pmol of the fluorescence-marked M13 primer and 0.15 units of recombinant GoTaq-polymerase (Promega, Charbonnieres, France). The PCR reactions were carried out in a 384-well plate. The PCR protocol was as follows: initial denaturation step at 94°C for 4 min, followed by 20 touch-down cycles involving denaturation at 94°C for 30s; annealing at 65–54°C for 45s and extension at 72°C for 30s; then 20 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45s and extension at 72°C for 30s; with a final extension step at 72°C for 2 min. To 1 µL of each PCR product was added 4.25µL of HiDiTM formamide and 0.25µL of GenScan-500 LIZ Size Standard (Applied Biosystems, Foster City, CA, USA). Electrophoresis and allele detection were carried out on an ABI3130xl Genetic Analyser (Applied Biosystems) with 36 cm capillaries. Output was analysed with Genemapper version 4.0 (Applied Biosystems). All marker data were verified manually by visual inspection. Because the orientation of the majority of the supercontigs is unknown, each SSR marker was consider to be located in the middle of the supercontig, in order to approximate the physical distance between markers.

RNAseq

RNA-seq analysis was carried out to compare the relative abundances of PAR gene transcripts at several different developmental stages of the life cycle (immature and fertile male and female gametophytes and several tissues of the sporophyte generation, namely basal filaments and upright filaments). The RNA extractions and processing of sequenced reads were performed as previously described in (5). Briefly, total RNA of young and mature gametophytes (near-isogenic male and female lines Ec603 and Ec602), basal and upright filaments of sporophyte-generation tissue were sequenced by Fasteris (CH-1228 Plan-les-Ouates, Switzerland) using Illumina HiSeq technology. Two biological replicates were sequenced per each library. Data quality was assessed using FASTX toolkit and the reads were trimmed and filtered by using a quality threshold of 25 (base calling) and a minimal size of 60bp. Only reads in which more than 75% of nucleotides had a minimal quality threshold of 20 were retained.

Filtered reads were mapped to the *Ectocarpus* sp. genome (40)(available at ORCAE (43)) using TopHat2 with the bowtie2 aligner (44). Reads mapped to exons were counted using HTSeq (45) and expression values were represented as RPKM (reads per kilobase per million mapped sequence reads). A filter of RPKM>1 was applied to remove noise and genes with very low expression levels.

Differential expression analysis between male and female gametophytes, as well as between gametophyte (males and females libraries as replicates) and parthenosporophyte was performed with the DESeq package (Bioconductor) (46) using an adjusted p-value cutoff of 0.1 and a minimal fold-change of 2. The PAR region was also analysed for the presence of duplicated genes. The clustering analysis was performed using MCL algorithm (Markov Cluster Algorithm) with the inflation value fixed to 3.0 and blastp with a minimal E-value set to 1e10-4.

Functional analysis

Ectocarpus genes were submitted to InterProScan (47) to recover functional annotations for each gene using Blast2GO (48). Fisher exact test with FDR corrected p-value cutoff of 0.05 (Blast2GO) were used to estimate the associations between GO-terms and genes with bias expression either between male/female gametophytes or between gametophyte/sporophyte generations.

Phase-specific gene expression

We used RNA-seq data representing four different life stages of *Ectocarpus* (male and female gametes, partheno-sporophytes, immature and mature gametophytes) and to two different tissue types (basal structures and upright filaments) to estimate breadth of gene expression. Gamete transcriptomic data was recovered from (49) and the expression values were represented as RPKM in order to make them comparable with other libraries.

The breadth of expression for each gene was measured using the tissue specificity index (τ) as described by (50):

$$\tau = \frac{\sum_{i=1}^{N}(1 - x_i)}{N - 1}$$

For each gene we calculated x_i as the expression profile in the given library *i* normalized by the maximal expression value across all analysed tissues (N). τ index values range from 0 to 1, where 1 implies strong tissue specificity of an investigated gene.

Evaluation of rates of gene evolution

To estimate evolutionary rates of PAR genes we searched *E. siliculosus* genomic data for orthologues by retaining best reciprocal Blastn matches with a minimum e-value of $10e^{-}10$. Sequences that produced a gapless alignment that exceeded 100bp were retained for pairwise dN/dS (ω) analysis using Phylogenetic Analysis by Maximum Likelihood (PAML, codeml, F3x4 model, runmode=-2). Genes with saturated synonymous substitution values (dS>1) and genes located in the sex-determining region were excluded from the analysis.

To detect PAR genes under positive selection we used transcriptomic and genomic data from four different *Ectocarpus* species as previously described in (51) (TableS3). These analyses were restricted to genes for which clear orthologues could be identified in the different species. Nucleotide alignments with minimum 100bp length for genes represented in all four investigated species were made using the ClustalW implemented in Mega6 (52, 53), curated manually when necessary and transformed to the PAML4 required format using perl fasta manipulation scripts (provided by Naoki Takebayashi, University Alaska Fairbanks).

Nonsynonymous (dN) and synonymous (dS) rates were estimated by the maximum likelihood method available in CODEML program (PAML4 package) using the F3X4 model of codon frequencies and a user tree specified according to the phylogeny. CODEML paired nested site models (M0, M3; M1a, M2a; M7, M8) (54) of sequence evolution were used in this analysis and compared using the likelihood ratio test (LRT). Empirical Bayes methods allowed for identification of positively selected sites a posteriori (55).

Effective Number of Codons (ENC) and Codon Adaptation Index (CAI) were calculated for all PAR and autosomal genes in this study using CAIcal server (http://genomes.urv.es/CAIcal/) (56).

Manual curation of genes located on linkages groups LG30 and LG04

To improve the comparison at the gene level between the LG30 and LG04, all genes were manually curated using RNA-seq data coverage and junction sites (available at ORCAE). Each gene was inspected to find missing exons, wrong start/stop or splice site and the structure was corrected.

Statistical analysis

We used Wilcoxon test to compare gene structural characteristics, gene expression levels, evolutionary rates, expression breadth and codon adaptation indexes. The distribution of generation biased genes among *Ectocarpus* chromosomes were calculated using a chi-square test with Bonferroni corrected p-values. All statistical analyses were performed in RStudio (R version 3.0.2).

Figures

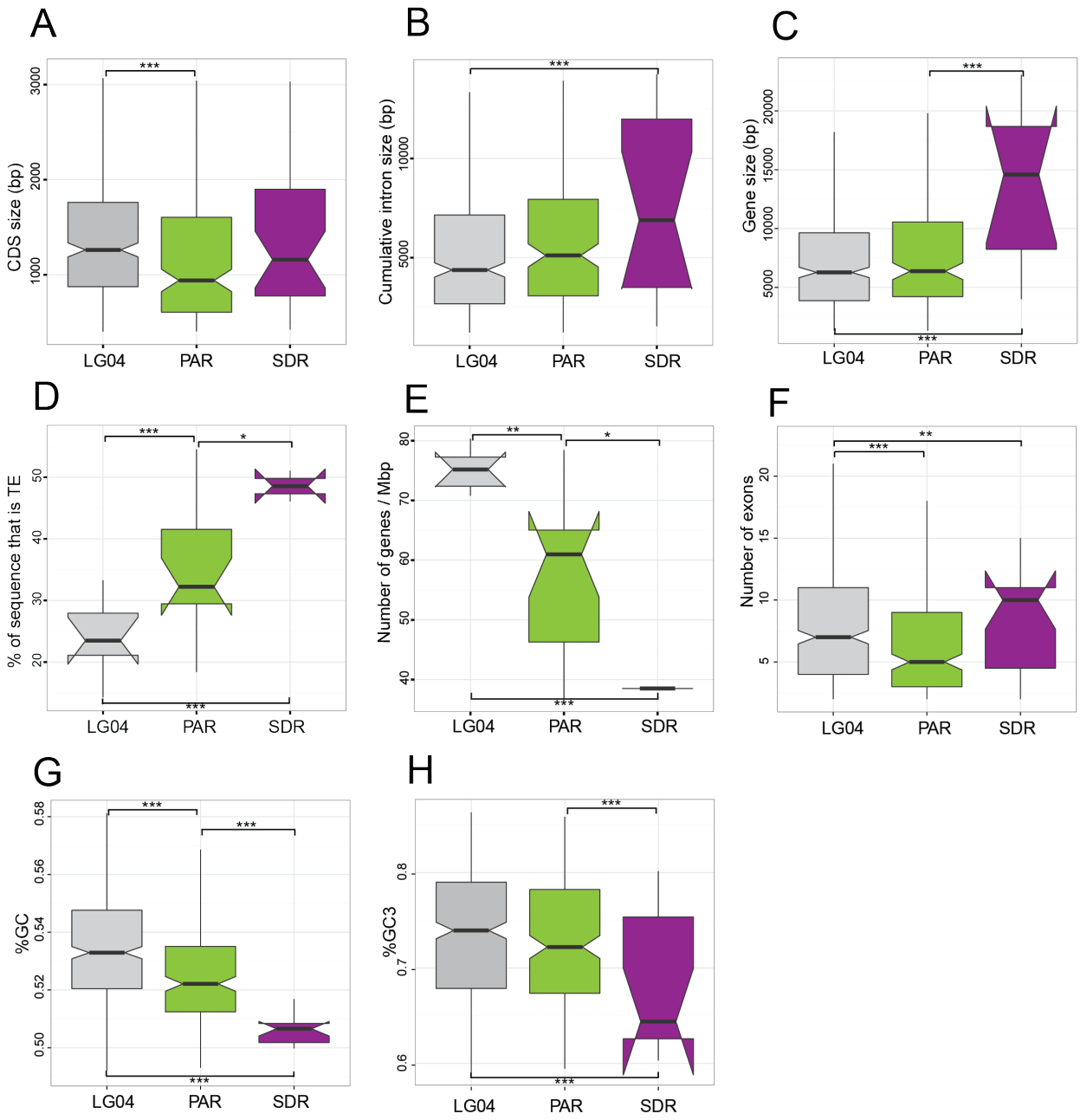


Figure 1. Luthringer & Lipinska *et al.* 2014

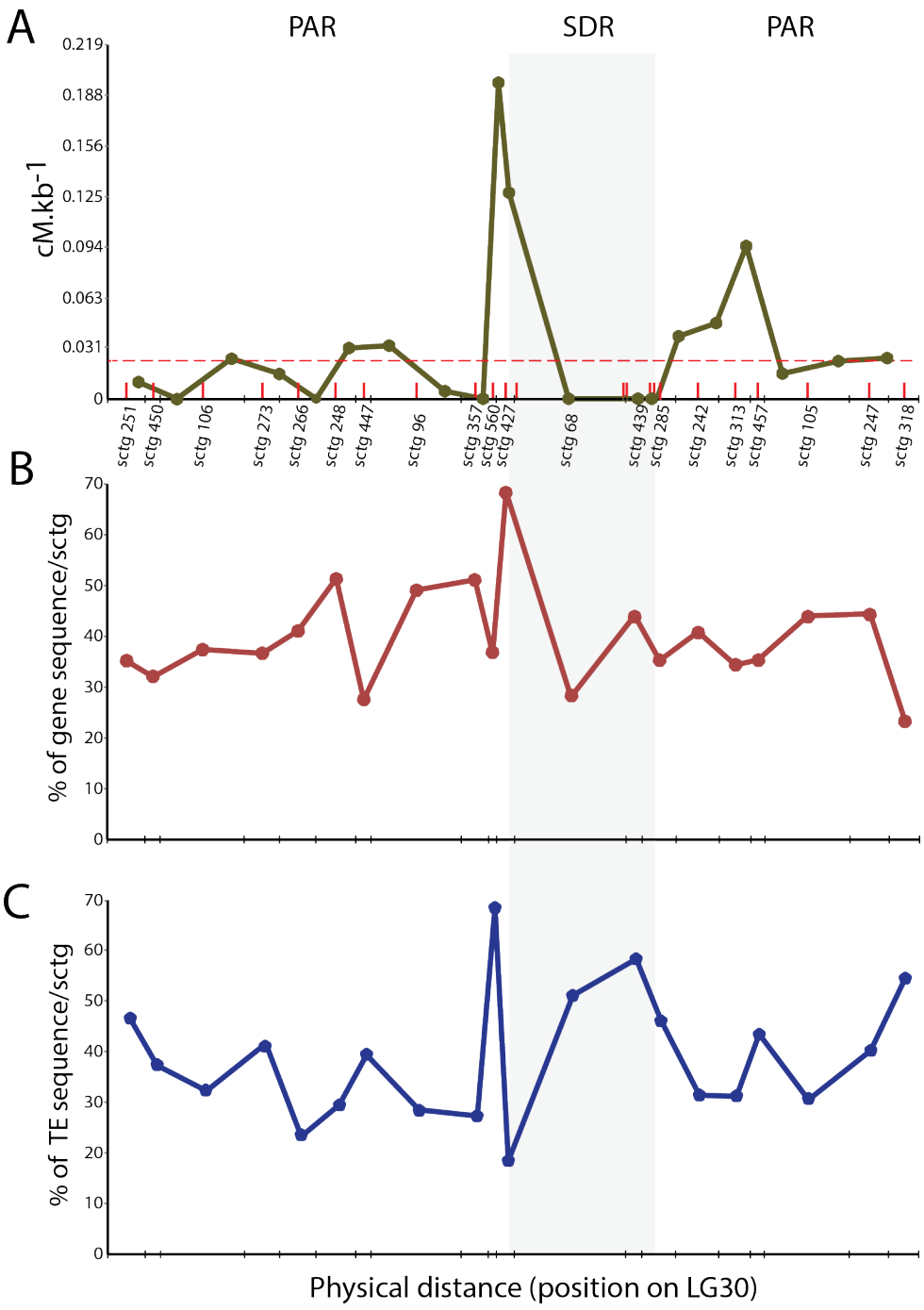


Figure 2. Luthringer & Lipinska *et al.* 2014

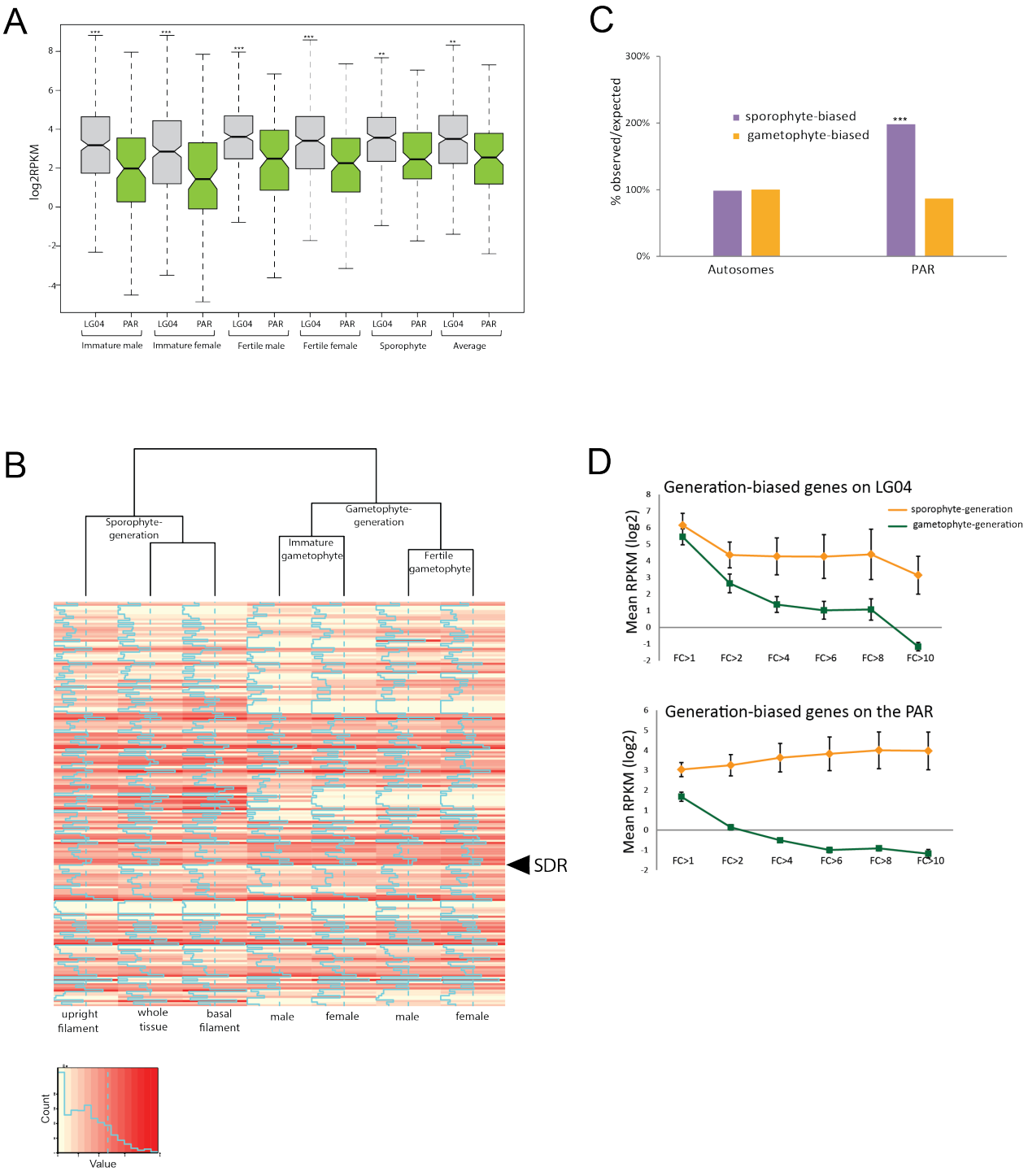


Figure3. Luthringer & Lipinska *et al.* 2014

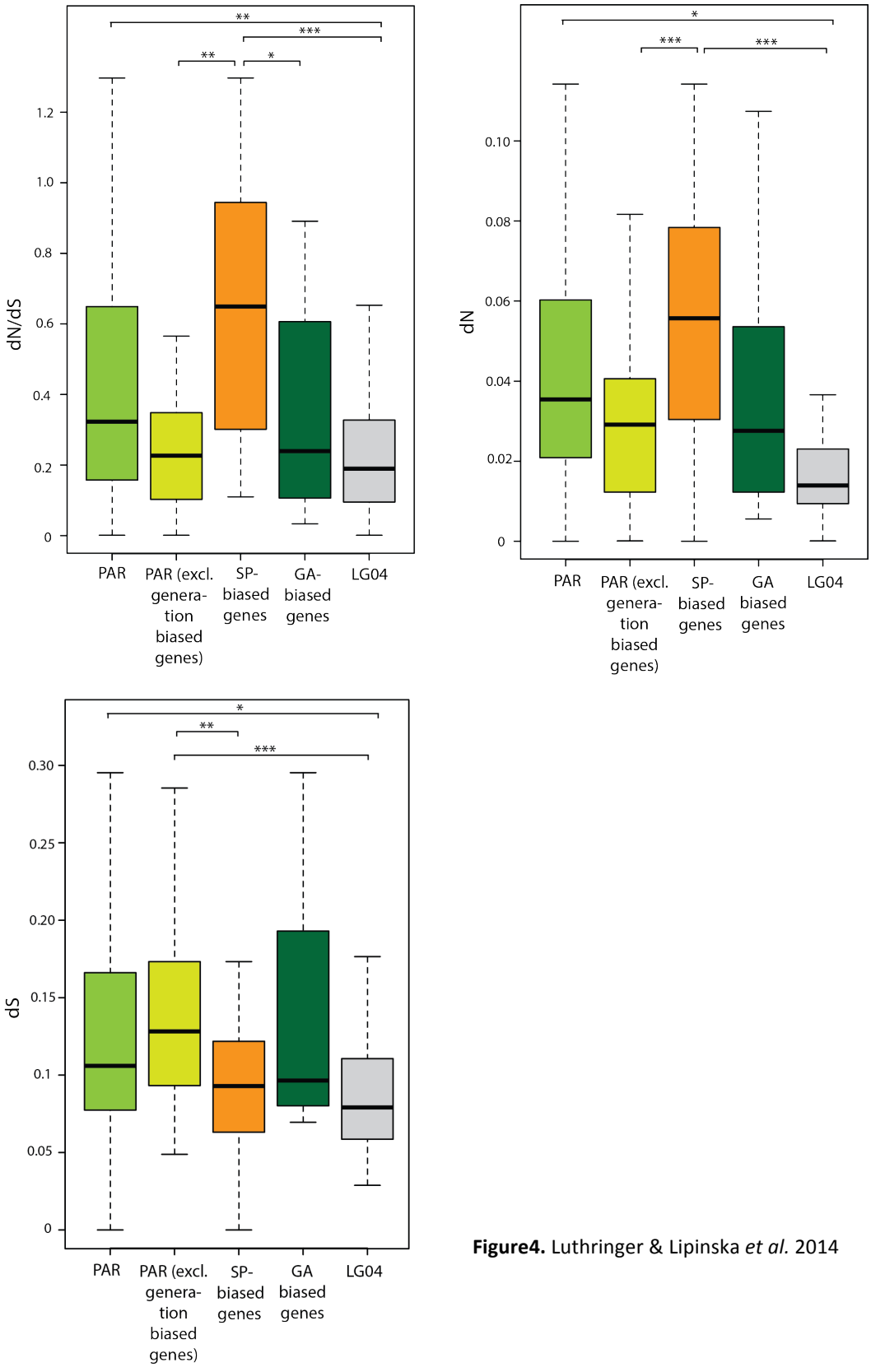


Figure4. Luthringer & Lipinska *et al.* 2014

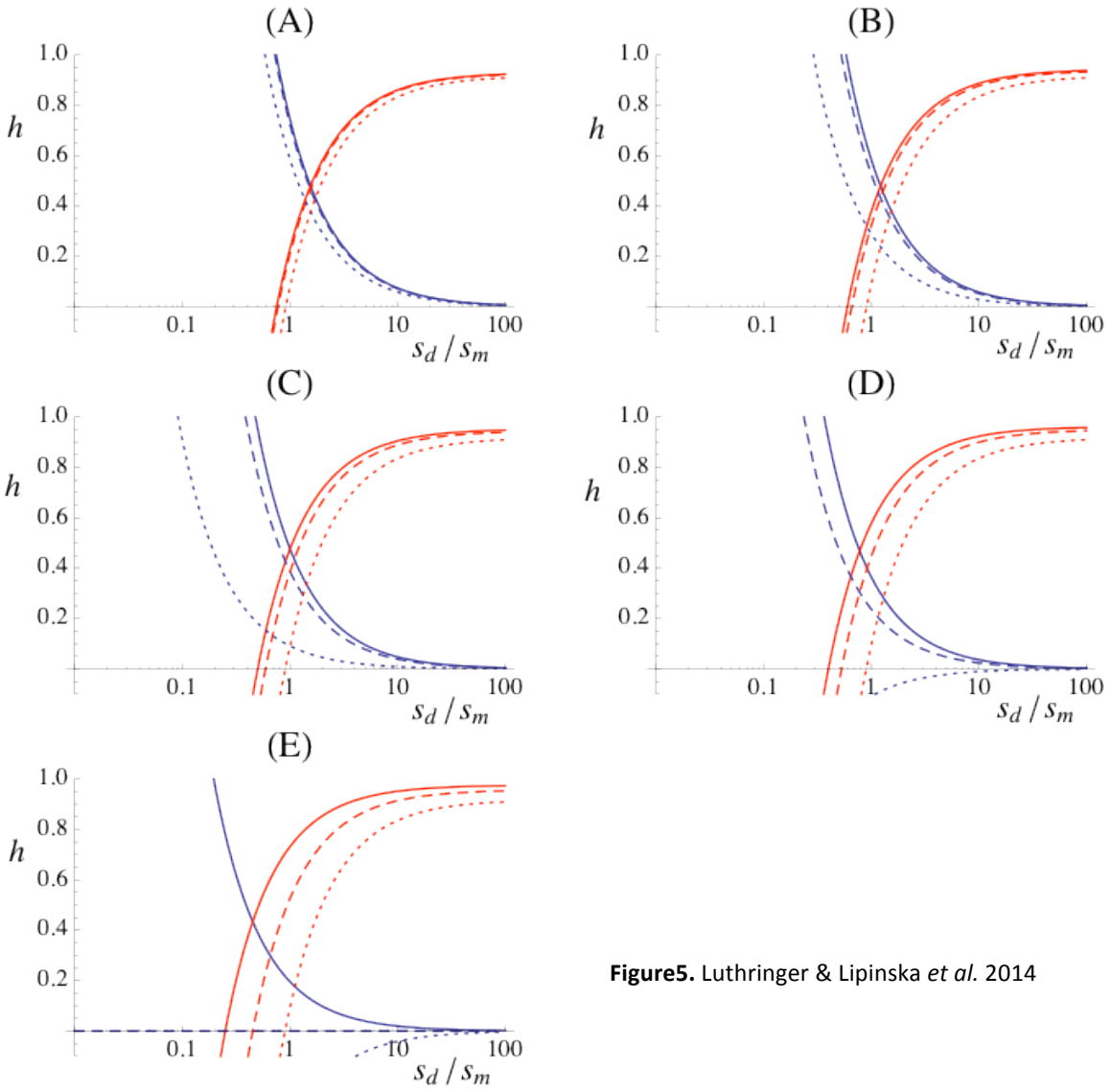


Figure5. Luthringer & Lipinska *et al.* 2014

FIGURE LEGENDS

Figure 1. **Structural characteristics of the PAR compared with the SDR and an autosome** (LG04). **A**) coding sequence (CDS) size **B**) total intron length **C**) gene size **D**) % TE **E**) gene density **F**) number of exons **G**) % GC **H**) %GC3.

Figure 2. Recombination frequency and distribution of transposable elements and gene density in the sex chromosome of *Ectocarpus*. A) The recombination frequency around the SDR is unusually high. The red dotted line represents the average recombination frequency over the entire *Ectocarpus* genome (42). The black and red lines on the x axis indicate boundaries between supercontigs (sctgs) and the mid points of supercontigs, respectively. **B** and **C**) Gene and transposable element (TE) density along the *Ectocarpus* sex chromosome. Analysis of gene and TE density was performed by calculating the % of bases on each supercontig that are part of a gene or a TE, respectively. Grey shading indicates the position of the non-recombining region (SDR).

Figure 3. Gene expression on the PAR during different life cycle stages. A) Average gene expression (RPKM) of PAR and autosomal (LG4) genes in male and female gametophytes (immature and fertile), and sporophytes. **B)** Heatmap of gene expression on *Ectocarpus* sex chromosome (excluding the sex determining region). Order of genes corresponds to physical location. **C)** Enrichment of sporophyte generation-biased genes on the PAR compared with autosomes and gametophyte generation-biased genes (Chi-square test, padj = $6.03e^{-5}$).

Figure 4. Rates of evolution of PAR (generation-biased and unbiased) genes compared with autosomal genes (LG4). Pairwise dN, dS and dN/dS ratios were calculated by comparing orthologous gene sequences from *Ectocarpus* sp. (lineage 1c) and *Ectocarpus siliculosus* (lineage 1a). **A**) Ratio of non-synonymous to synonymous substitutions (dN/dS). **B** and **C**) Non-synonymous substitutions (dN) and synonymous substitutions (dS).

Figure 5. Effect of linkage to the sex locus on the spread of alleles *a* and *A* under different conditions.

Supplemental Data

Model

Alleles *F* and *M* (for female and male) segregate at the first locus, fertilization being possible between *F* and *M* gametes only. We denote *A* and *a* the alleles at the second locus, and write the fitness of the different haploid and diploid genotypes as in Table S4. s_d measures the selective advantage of allele *a* in diploids and *h* its dominance coefficient, while s_f and s_m measure the advantage of allele *A* in females and males, respectively (note that these coefficients may be negative). The average selection coefficient in haploids is denoted by $s_h = (s_f + s_m)/2$, while $d_{fm} = (s_f - s_m)/2$ measures the difference in selection between males and females (we thus have $s_f = s_h + d_{fm}$ and $s_m = s_h - d_{fm}$). Finally, *r* is the recombination rate between the sex-determining locus and the selected locus: $r < 0.5$ thus means that the selected locus is located in the PAR.

	AA	Aa	aa	A	a
diploid	1	$1 + h s_d$	$1 + s_d$		
female				$1 + s_f$	1
male				$1 + s_m$	1

Table S4. Fitnesses of the different genotypes at the selected locus.

In the following, x_1, x_2, x_3 and x_4 denote the frequencies of *MA*, *Ma*, *FA* and *Fa* individuals at the start of the haploid generation. Frequencies at the next generation (after haploid selection, random fusion between *F* and *M* gametes, diploid selection and recombination) are given by (see *Mathematica* file for derivation):

$$T x_1' = (1+s_f)(1+s_m)x_1x_3 + (1+h s_d)\left[r(1+s_f)x_2x_3 + (1-r)(1+s_m)x_1x_4\right]$$

$$T x_2' = (1+s_d)x_2x_4 + (1+h s_d)\left[r(1+s_m)x_1x_4 + (1-r)(1+s_f)x_2x_3\right]$$

$$T x_3' = (1+s_f)(1+s_m)x_1x_3 + (1+h s_d)\left[r(1+s_m)x_1x_4 + (1-r)(1+s_f)x_2x_3\right]$$

$$T x_4' = (1+s_d)x_2x_4 + (1+h s_d)\left[r(1+s_f)x_2x_3 + (1-r)(1+s_m)x_1x_4 \right]$$

with:

$$T = 2\left[(1+s_f)(1+s_m)x_1x_3 + (1+s_d)x_2x_4 + (1+h s_d)\left[(1+s_f)x_2x_3 + (1+s_m)x_1x_4 \right] \right].$$

Through the following we assume that s_d and s_h are both positive: allele *a* is beneficial for diploids, while *A* is on average beneficial for haploids (although the strength and direction of selection may differ among sexes, *i.e.*, s_f and s_m may be different and may have opposite signs). The diploid-beneficial allele (*a*) increases in frequency when rare when the leading eigenvalue associated with the equilibrium $(x_1, x_2, x_3, x_4) = (0.5, 0, 0.5, 0)$ is greater than 1. This eigenvalue is given by:

$$\lambda_a = \frac{1+h s_d}{(1+s_f)(1+s_m)}\left[(1-r)(1+s_h) + \sqrt{d_{fm}{}^2(1-2r) + r^2(1+s_h)^2} \right].$$

Similarly, the haploid-beneficial allele (*A*) increases when rare when the leading eigenvalue λ_A associated with the equilibrium $(x_1, x_2, x_3, x_4) = (0, 0.5, 0, 0.5)$ is greater than 1, where:

$$\lambda_A = \frac{1+h s_d}{1+s_d}\left[(1-r)(1+s_h) + \sqrt{d_{fm}{}^2(1-2r) + r^2(1+s_h)^2} \right].$$

From these expressions, one obtains that linkage to the sex-determining locus does not affect conditions for the spread of both *a* and *A* alleles when selection does not differ between sexes ($d_{fm} = 0$). Indeed, in this case the expressions for λ_a and λ_A do not depend on *r*, and simplify to:

$$\lambda_a = \frac{1+h s_d}{1+s_h}, \quad \lambda_A = \frac{1+s_h}{(1+s_d)/(1+h s_d)}.$$

That is, allele *a* spreads when its benefit for diploids ($h s_d$, since *a* is mostly expressed in heterozygotes as long as it is rare) is higher than its deleterious effect for haploids (s_h). Conversely, *A* spreads from rarity when its benefit for haploids (s_h) is greater than its cost for diploids, which depends on the relative fitness of *aa* and *Aa* individuals, since the frequency of *AA* individuals is negligible as long as *A* is rare.

When selection differs among sexes ($d_{fm} \neq 0$), however, linkage to the sex-determining locus affects conditions for spread of ploidally antagonistic alleles. From the expressions of λ_a and λ_A given above, one obtains that both *a* and *A* benefit from linkage to the sex locus when they are rare in the population. This can be seen most easily in the limit where *r* tends to zero (complete linkage): in this case, the expressions for λ_a and λ_A simplify to:

$$\lambda_a = \frac{1 + h\,s_d}{1 + \mathrm{Min}\left[s_f, s_m\right]} \quad , \quad \lambda_A = \frac{1 + \mathrm{Max}\left[s_f, s_m\right]}{\left(1 + s_d\right) / \left(1 + h\,s_d\right)}$$

where $\mathrm{Min}\left[s_f, s_m\right]$ and $\mathrm{Max}\left[s_f, s_m\right]$ are the minimum and maximum of s_f and s_m. Consider for example the case where alleles *A* and *a* are neutral in males ($s_m = 0$): if the diploid-beneficial mutation *a* occurs in complete linkage with the male-determining allele *M*, its deleterious effect in females is never expressed and therefore does not prevent its increase in frequency (until all males carry *a*, and thus all diploids are *Aa*). Conversely, if the female-beneficial allele *A* occurs in complete linkage with the female-determining allele *F*, it benefits from a stronger increase in frequency during the haploid phase than if it was freely recombining with the sex-determining locus, in which case its change in frequency would be determined by its average effect over both sexes (indeed, λ_A simplifies to $\left(1 + s_h\right) / \left[\left(1 + s_d\right) / \left(1 + h\,s_d\right)\right]$ when $r = 1/2$). The same effects occur when $s_m \neq 0$, the effect of linkage to the sex locus being more marked as the difference between s_f and s_m increases. Finally, note that under our assumption of ploidally-antagonistic selection (s_d, $s_h > 0$), linkage to the sex locus may yield higher benefits to diploid-beneficial alleles than to haploid-beneficial alleles, since the effect of haploid selection may be entirely suppressed (when s_f or s_m equals zero), or even reversed when selection is sex-antagonistic − by contrast, haploid-beneficial alleles always decrease in frequency during diploid selection.

Table S2. See attached Excel file "Supplemental Luthringer & Lipinska *et al*. 2014" sheet "Table S2"

Feature	S	rho	p-value	FDR	padj (Bonferroni)
GC	1549098	-0,01812531	0,7945	0,8938125	1
GC3	1730323	-0,1372332	0,04754	0,21393	0,42786
gene.size	1512294	0,006063683	0,9306	0,9306	1
cds.size	1393622	0,08405947	0,2262	0,50895	1
%geneTE	1612243	-0,05962663	0,3911	0,70398	1
intron size	1789376	-0,1760451	0,01078	0,09702	0,09702
exon size	1471029	0,03318469	0,6334	0,8938125	1
Av_log2RPKM	1703404	-0,119541	0,08471	0,25413	0,76239
tissue sp	951568	0,02099036	0,7795	0,8938125	1

Table S1. Luthringer & Lipinska *et al*. 2014

Species/Lineages	Strain reference	Isolation location
Ectocarpus siliculosus 1a	Rb1 x EA1 progeny	Naples, Italy
Ectocarpus sp. Peru 1c	Ec602, Ec603, Ec32	Peru
Ectocarpus sp. Greenland 1c	CCAP 1310/214	Kapisigdlit, Godhåbsfjorden, West Greenland
Ectocarpus fasciculatus	CCAP 1310/13	Roscoff, France
Scytosiphon lomentaria	Slom	Asari, Japan

Table S3. Luthringer & Lipinska *et al*. 2014

	AA	Aa	aa	A	a
diploid	1	$1 + h\,s_d$	$1 + s_d$		
female				$1 + s_f$	1
male				$1 + s_m$	1

Table S4. Luthringer & Lipinska *et al*. 2014

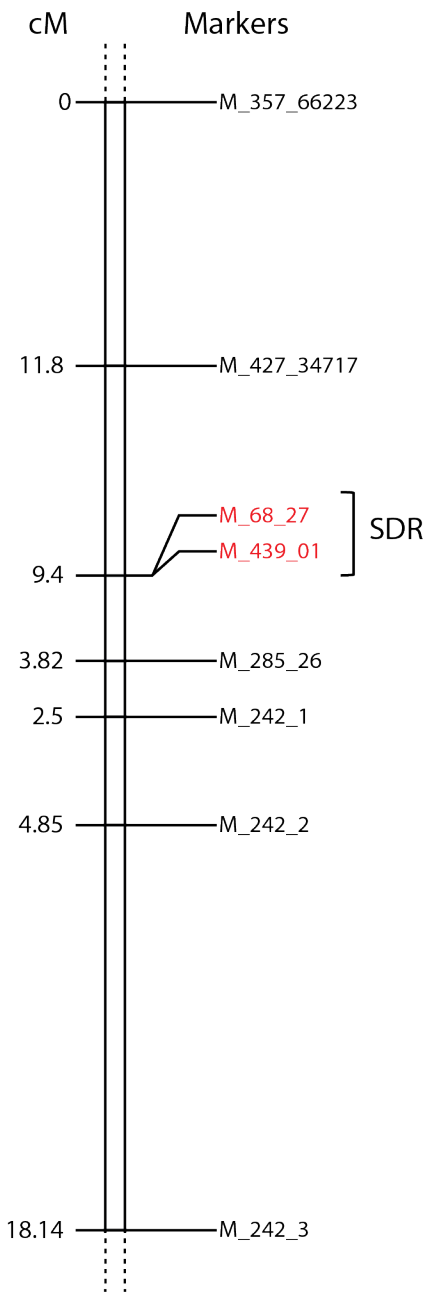


Figure S1. Luthringer & Lipinska *et al.* 2014

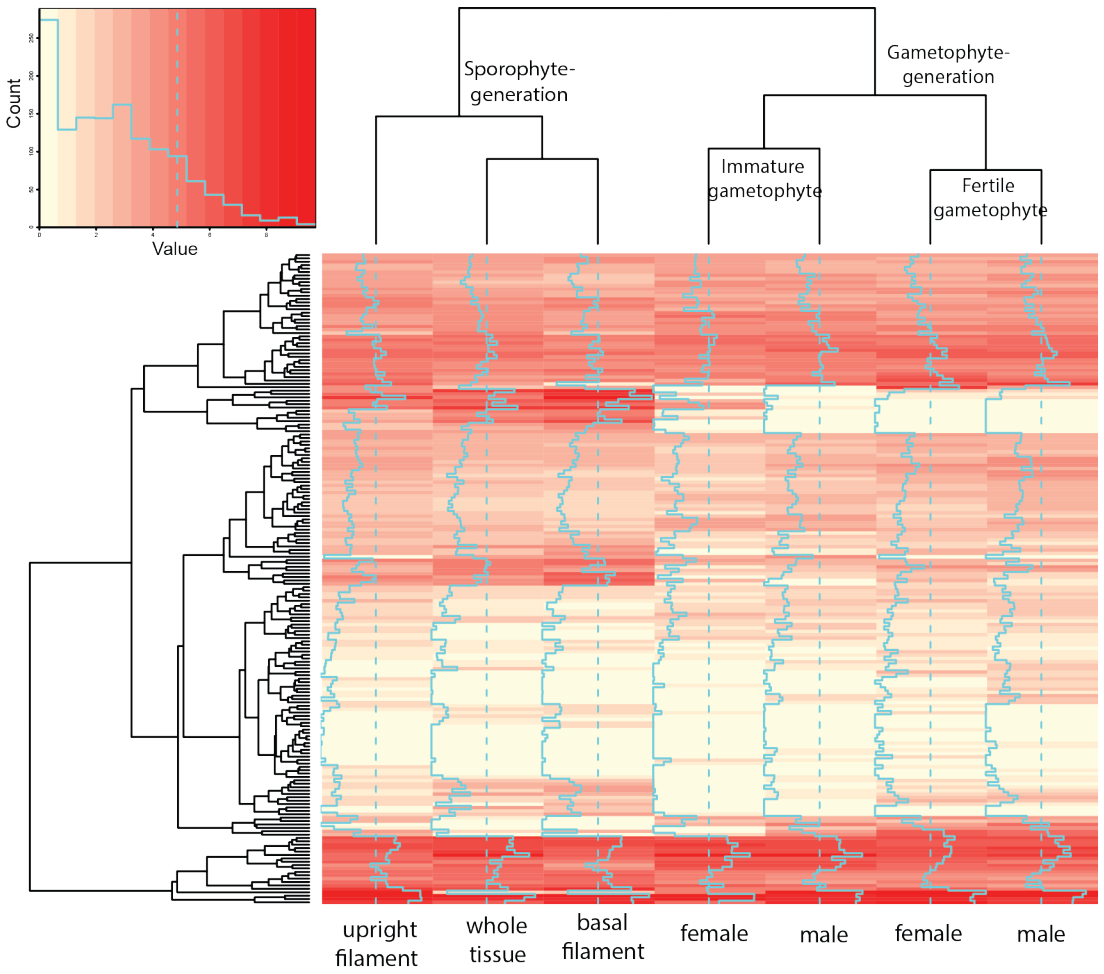
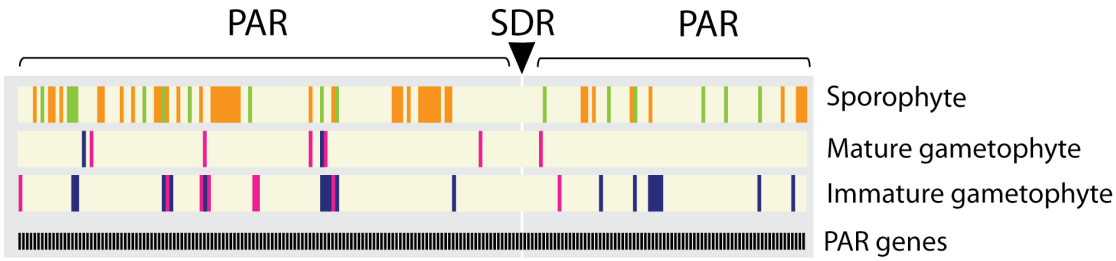


Figure S2. Luthringer & Lipinska *et al.* 2014

A



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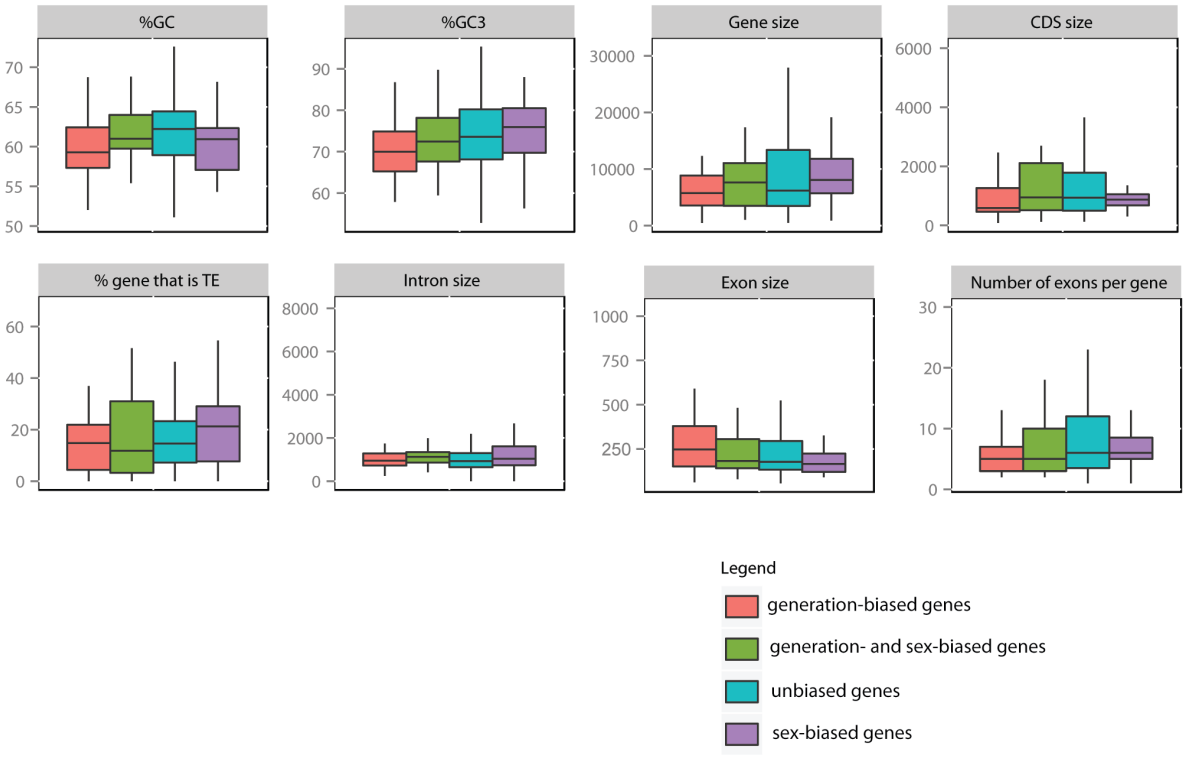


Figure S3. Luthringer & Lipinska *et al.* 2014

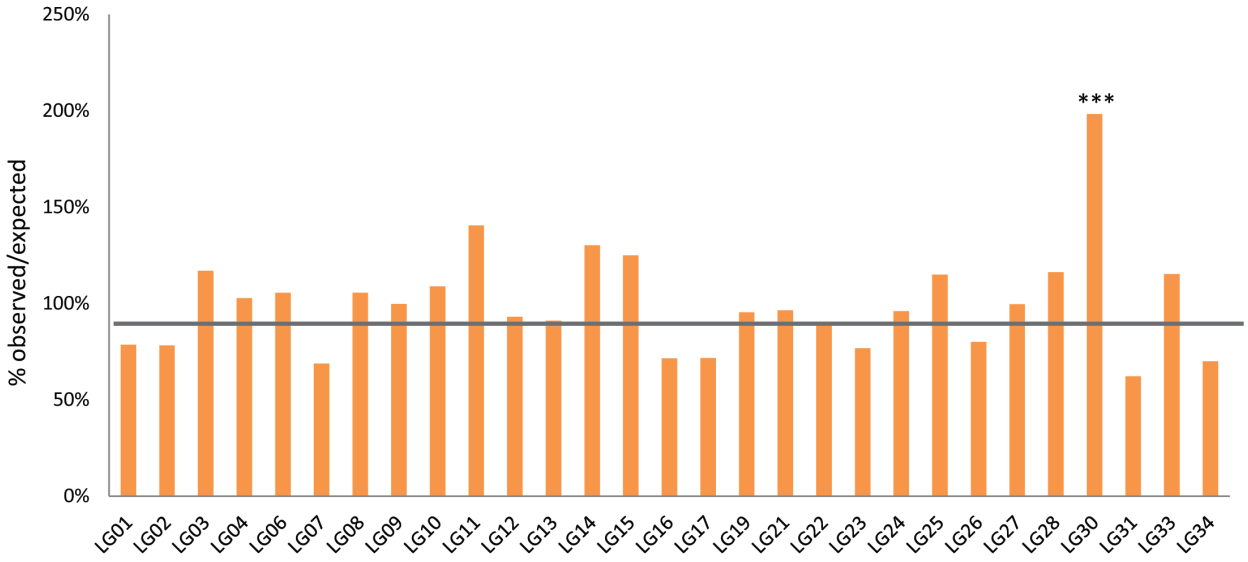
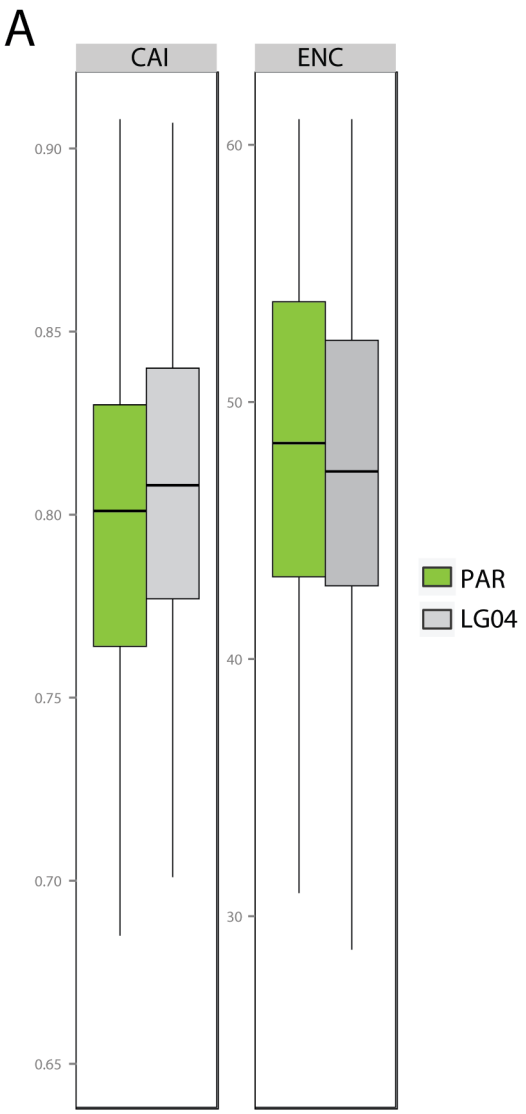


Figure S4. Luthringer & Lipinska *et al.* 2014



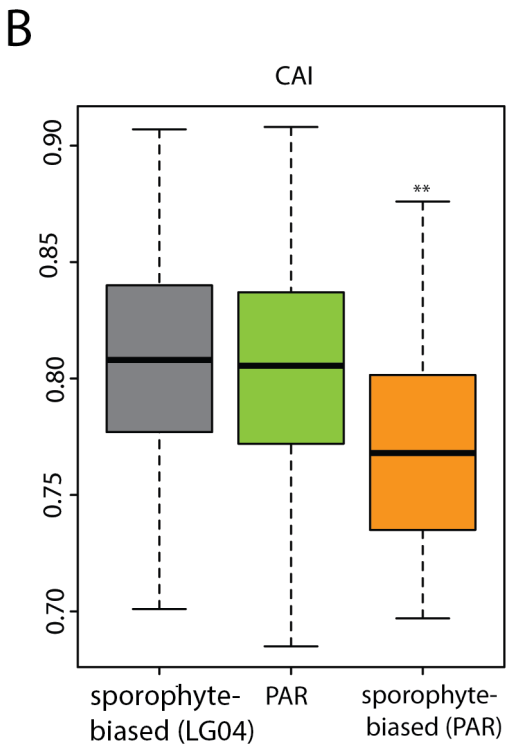


Figure S5. Luthringer & Lipinska *et al.* 2014

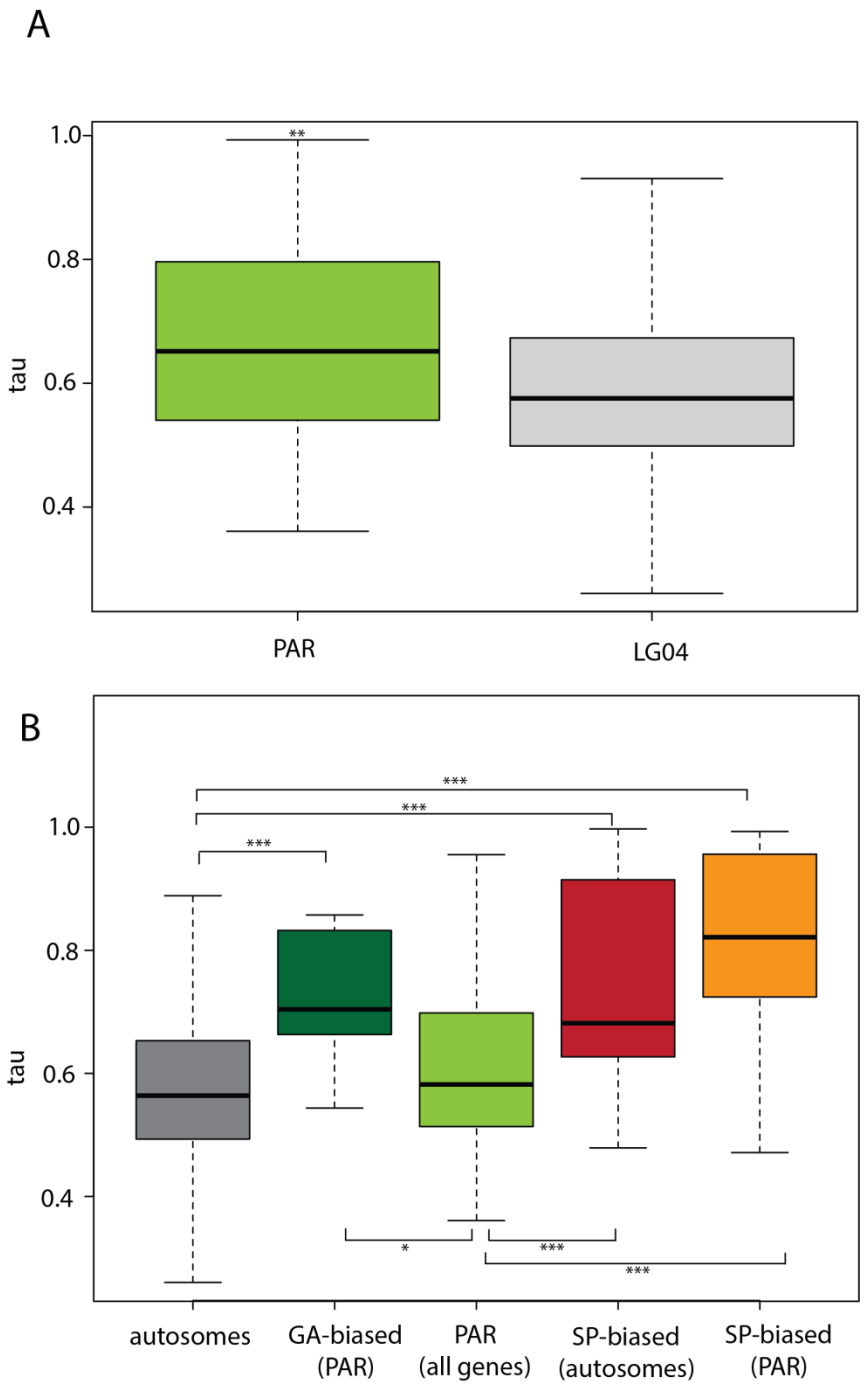


Figure S6. Luthringer & Lipinska *et al.* 2014

Supplementary data:

Table S1. Correlation of PAR structural characteristics in relation to the distance to the SDR (Spearman's rho; p_{adj} with Bonferroni correction).

Table S2. List of genes on the PAR with corresponding functional annotation and expression bias information.

Table S3. *Ectocarpales* species used in the study.

Table S4. Fitnesses of the different genotypes at the selected locus.

Figure S1. **Recombination events in the PAR of *Ectocarpus siliculosus* (lineage 1a) species**. Genetic linkage map for the sex chromosome of *E. siliculosus lineage 1a*. The positions of simple sequence repeat (SSR) markers are indicated to the right of each linkage group, with the prefix 'M' for marker followed by the number of the supercontig that contains the SSR. Numbers to the left indicate the map distances (in cM) between the intervals given by the lines that cross the vertical bar. The SSR markers corresponding to the sex determining region (SDR) are marked in red.

Figure S2. Heatmap of gene expression on the PAR, clusters according to the expression levels. Names of genes have not been included for simplicity.

Figure S3. **A**) Sex-biased and generation biased genes on the PAR. Genes are represented by coloured bars according to the physical location on the PAR (SDR excluded). Pink bars – female-biased genes; Blue bars – male-biased genes; Orange bars – sporophyte-generation biased genes; Green bars – gametophyte-generation biased genes; Beige bars – unbiased genes. **B**) Generation- and sex- biased genes on the PAR show no significant differences in structural characteristics. Mixed-biased genes – genes exhibiting sex- and generation- biased expression.

Figure S4. **Distribution of sporophyte biased genes on the *Ectocarpus* chromosomes**. Sporophyte-biased genes are significantly enriched on the sex chromosome (LG30) (Chi-square test with Bonferroni correction, $p<0.001$).

Figure S5. Average codon usage bias on the PAR. **A**) Codon adaptation index (CAI) and effective number of codons (Nc) for the PAR and autosomal (LG4) genes show no significant differences between the groups (Wilcoxon test, $p=0.318$). **B**) Codon adaptation index (CAI) is significantly lower for the sporophyte biased genes on the PAR compared to the autosomal (LG4) and other PAR genes (Kruskal-Wallis with Dunn's post-test, $p<0.001$).

Figure S6: Expression breadth of PAR genes. A) Expression breadth of all PAR genes and LG04 genes. **B**) Generation-biased genes expression breadth.

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III. Discussion and perspectives

The female and male non-recombining regions in the *Ectocarpus* sex chromosomes have an unusual genomic structure compare to autosomes, with low gene density, high density of repeats and transposable elements (TE) and low GC content (Ahmed *et al*., 2014). These unusual features are however not restricted to the non-recombining region, the PAR has TE density, gene densities and GC contents that are intermediate between those for the SDR and the autosomes. In other systems, these parameters have been correlated with the level of recombination, with a positive correlation observed between recombination rate on the one hand and gene density and GC content on the other. In addition, recombination rate has been negatively correlated with TE density (Chen *et al*., 2006; Fullerton *et al*., 2001; Tian *et al*., 2009, 2012) The analysis of the recombination map on the *Ectocarpus* sex chromosome reveals that, despite the two hotspots flanking the SDR, both PARs have the same average recombination rate as autosomes. We can therefore conclude, that the unusual genomic features observed in the PARs cannot be explained by the level of recombination. Then how can we justify the weak proportion of genes and the high density of TEs in the PARs?

Several hypotheses can be put forward:

1) In other systems, it has been observed that a certain class of TE have preferential sites of insertion. In *A. thaliana* a non-uniform distribution of some retrotransposons is found in the pericentromeric region of chromosomes. This distribution was hypothesized to be the result of several factors including target-site bias, accumulation in non-recombining regions (pericentromeric) and selection against insertion in gene-rich region (euchromatin) (Pereira, 2004; Peterson-Burch *et al*., 2004). The latter factor can also explain the density of TEs in the *Ectocarpus* PAR, which has a low density of genes compared to autosomes (Ahmed *et al*., 2014) and may therefore be a preferential region of TE insertion. Indeed gene poor genomic regions can preferentially receive TE because there is less chance of insertions causing harmful mutations (Boeke and Devine, 1998). Under this hypothesis, we have to assume that the decrease in gene density predates the increase of TE in PAR.

2) The PAR is an ancient non-recombining region that subsequently restarted to recombine. One possible explanation is that all or a large proportion of the sex chromosome was non-recombining and then recombination was restored to finally establish a non-recombining region of 1 Mbp. Under this “SDR contraction” hypothesis, from a presumably identical ancestral SDR, restoration of recombination should have occurred independently in

different brown algal species and therefore orthologues of *Ectocarpus* PAR genes could be sex-linked in some brown algae and not in others. If this hypothesis is correct, then we would expect to find orthologs of *Ectocarpus* PARs genes that would be sex-linked in other brown algae, and possibly orthologs of *Ectocarpus* SDR genes in the PAR of other browns.

Interestingly, we found an ortholog of an *Ectocarpus* gene (*Esi0285_0026*) that is located near the border of the SDR in *Ectocarpus* but is sex-linked in a Laminariales species, *Undaria pinnatifida*. The position of this gene on the border of the *Ectocarpus* SDR supports the second hypothesis. Alternatively, this gene may have been under sexually antagonistic selection, and was therefore subsumed into the SDR of *Undaria* after the separation of the two species, *i.e*. differential expansion of the non-recombining region in *Ectocarpus* and *Undaria*. *Undaria* has female and male gametophytes that are highly dimorphic and produces oogamous gametes. This species therefore displays a higher level of sexual dimorphism than *Ectocarpus*. If sexual conflict in *Undaria* is stronger than *Ectocarpus*, this could lead to stronger forces to include genes under SA selection in the SDR, as genes of this type are predicted to accumulate just outside the SDR (Charlesworth, Jordan, & Charlesworth, 2014; Otto *et al*., 2011 and model in this paper). To test those hypotheses, searches need to be carried out for orthologs of other *Ectocarpus* PAR genes from around SDR to determine whether they are sex-linked in other brown algae, specifically in species where sexual dimorphism is stronger than in *Ectocarpus*. The order Laminariales is suitable for such a study because there is now convincing evidence that sex chromosomes of the Ectocarpales and the Laminariales share the same origin (Ahmed *et al*., 2014).

The recombination map of the *Ectocarpus* PARs presented in this study was constructed for the genome of the sequenced strain from Peru (*Ectocarpus* 1c lineage, Stache-Crain *et al*., 1997). We also analysed recombination rates around the SDR in another species of *Ectocarpus* from Naples (*i.e*. *Ectocarpus siliculosus stricto sensu,* 1a lineage) to investigate conservation of recombination in PARs in other species of *Ectocarpus* (Figure S1 in this chapter section II). Future work on *Ectocarpus* PARs could construct and analyse a physical map for *E. siliculosus* and construct a recombination map to analyse the conservation of hotspots of recombination in different species of *Ectocarpus*. A recent study of the human PAR1 showed that different human populations do not share the same hotspots of recombination on the PARs (Hinch *et al*., 2014).

Such an analysis of recombination within sex chromosomes in different populations and species of *Ectocarpus* with different life history traits could be an interesting further

study. Indeed, differences in life history traits probably generate different selective pressures, including in terms of sexual selection. For instance, samples of *E. siliculosus* from Naples include principally the haploid gametophytic stage, *i.e.* in the sexual generation of the life cycle. In contrast, the sequenced Peruvian strains from which sex chromosomes were identified and analysed (Ahmed et al 2014) are found more commonly in the diploid sporophytic generation (Alejandro Montecinos personal communication). A population that spends most of the time in the sexual stage (gametophytic generation) would probably be under stronger sexual selection than a population that is more often in the non-sexual stage (sporophytic generation). Therefore, such differences in life history traits could induce differences in the strength of sexual selection and influence the evolution of the non-recombining SDR and this could provide a unique opportunity to analyse an evolving SDR in a UV system.

Chapter 4. Evolution of Sex-Biased Gene Expression in a Haploid Sex-Determination System with Limited Sexual Dimorphism

I. Introduction

Many traits may have different fitness optima in males and females and this may lead to sexual conflict. The genetic basis of those differences between females and males can generate conflict known as intralocus sexual conflict. Such a conflict can arise when an allele is favourable for one sex but harmful for the other, resulting in genetic sexual antagonism between sexes. There are several ways to solve this conflict: by gene duplication and subfunctionalisation (Connallon and Clark, 2011), by alternative splicing to generate male- and female-advantageous transcripts (Stewart *et al*., 2010), by modulating expression so the gene is only expressed in the sex that it benefits, or finally, if the sexual antagonistic (SA) gene is closely linked to the SDR, loss of recombination could be favoured in order to associate the allele with the sex that is benefits (see Chapter 1 section II.d). Modulation of expression to resolve sexual antagonism, *i.e*. sex-biased gene (SBG) expression, has been broadly studied in diverse organisms. Using SBG expression as a proxy for resolved sexual antagonism provides access to genes that were under SA and probably to genes that control sex differences. Also is should be noted that there is some limit in the use of SBG as a proxy for resolved SA (Innocenti and Morrow, 2010; Mank, 2009; Parsch and Ellegren, 2013).

Rice (1984) predicted that SA alleles should be unevenly distributed in the genome. This prediction arose from the observation that sex chromosomes do not spend the same amount of time in each sex. Models based on the difference of inheritance of sex chromosomes predict that sex-specific sex chromosomes (Y and W) should accumulate alleles that are beneficial to the heterogametic sex (male and female in XY and ZW systems respectively). The X and Z are expected to accumulate beneficial alleles for both sexes, but under different dominance regimes, *i.e*. recessive alleles that benefit the heterogametic sex and dominant alleles that benefit the homogametic sex. Therefore non-degenerated homomorphic sex-chromosomes (X and W), are expected to be hot-spots of SA polymorphism and consequently hotspots of SBGs. Some empirical data tend to confirm

those predictions. For instance such a pattern of SBG distribution has been found in human, mouse, *D. melanogaster*, chicken, emu, *S. latifolia* and *C. elegans* (Assis *et al*., 2012; Connallon and Jakubowski, 2009; Gibson *et al*., 2002; Jaquiéry *et al*., 2013; Kaiser and Ellegren, 2006; Khil *et al*., 2004; Lercher *et al*., 2003; Parisi *et al*., 2003; Pischedda and Chippindale, 2006; Qiu *et al*., 2013; Reinke *et al*., 2004; Vicoso *et al*., 2013b). Nevertheless some analyses have shown that sexual antagonism can also be found in autosomes (Calsbeek and Sinervo, 2003; Delcourt *et al*., 2009; Fedorka and Mousseau, 2004), as predicted by Fry's model (Fry, 2010).

Another interesting aspect of SBGs resides in their evolutionary fate. Gamete recognition proteins show rapid evolution (Vacquier, 1998) and it has been proposed that genes with sex-specific expression evolve more rapidly (Haerty *et al*., 2007). This rapid evolution may be the result of several selective forces in each sex. The first occurs when numerous male gametes compete to have access to the fewer, large female gametes, known as sperm competition. The second force is sexual selection due to mate choice, this choice is often made by the female. Finally when male and female interests are different, as described in the previous paragraph with sexual antagonism, there is a sexual conflict, which can drive the rapid evolution of sex-genes (Swanson and Vacquier, 2002). Two of these three forces, sperm competition and sexual selection, preferentially affect males, who are therefore more prone to selective forces, explaining why genes involved in male traits and reproduction evolve faster than female and autosomal genes (Grath, 2010; Grath and Parsch, 2012; Jagadeeshan and Singh, 2005; Meiklejohn *et al*., 2003; Perry *et al*., 2014; Pointer *et al*., 2013; Zhang *et al*., 2007). Because of their role in sexual reproduction, SBGs are expressed during a specific phase of a life cycle and or in a specific tissue, therefore often SBG have reduced breath of expression which is also know to induce a rapid evolution of protein-coding genes (Duret and Mouchiroud, 2000; Haerty *et al*., 2007). However, some patterns of SBGs evolution are unexpected, such as in birds where female-biased, brain-expressed genes evolve faster than male-biased genes expressed in the brain. Several hypothesis were proposed to explain such a pattern, among them the possibility that the high dN/dS values observed for female-biased genes were the result of relaxed natural selection and, conversely, in males purifying selection has led to low dN/dS values (Mank *et al*., 2007).

Most of our knowledge about SBG expression and evolution was generated by the analysis of animals and plants and nothing is known about SBGs in UV systems. Recently, two studies analysed sex-specific expression of genes in brown algae. The first was conducted

on *Fucus vesiculosus*, a dioecious brown alga. This study confirmed the general trend observed, that there are more genes expressed preferentially in males than in females, respectively 14% and 9% (Martins *et al*., 2013). The second study analysed sex-biased expression in *Ectocarpus* gametes where 51.6% and 49.4% of the SBGs were preferentially expressed in male and female gametes respectively (Lipinska *et al*., 2013).

The aim of the analysis presented in this chapter was to understand the pattern of expression, the genomic location and evolutionary pattern of SBG in the *Ectocarpus* UV system. This analysis of SBGs was also expected to help explaining why the *Ectocarpus* SDR size has remained so small despite being old (Ahmed *et al*., 2014; see Chapter 2).

II. Paper

Title:

Sexual dimorphism and the evolution of sex-biased gene expression in the brown alga *Ectocarpus*

(Article submitted to *Molecular Biology and Evolution*)

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R.L. contributed to this paper by preparing *Ectocarpus* cultures, performing genetic crosses, sexing individuals, identifying sexual dimorphic traits in *Ectocarpus* and participating in the transcriptomic data analysis, specifically the bioinformatics analysis of the *Scytosiphon lomentaria* transcriptome.

ABSTRACT

Males and females have often marked phenotypic differences, and these are thought to result from sex differences in gene expression. Sex-biased gene expression has been well characterized in animal species, where a high proportion of the genome may be differently regulated in males and females during development. Genes with male-biased expression have been associated with rapid gene divergence, implying that selective differences exist between sexes. These investigations have focused on organisms exhibiting separate sexes during the diploid phase of the life cycle (diploid sexual systems), but the genetic nature of the sexual system is expected to influence the evolutionary trajectories of sex-biased genes. We analyse here the patterns of sex-biased gene expression in *Ectocarpus*, a brown alga displaying haploid sex determination (dioicy) and low phenotypic sexual dimorphism. We found that *Ectocarpus* is an exceptional example of an organism where female-biased genes evolve as rapidly as male-biased genes. We reveal the complex pattern of evolution for sex-biased genes throughout gametophyte development, with genes expressed at fertility showing faster evolutionary rates. Both male and female-biased genes had a greater portion of sites experiencing positive selection, suggesting that their accelerated evolution is at least partly driven by adaptive evolution. Gene duplication may underlie the generation of sex-biased gene expression, expanding previous models that propose this mechanism for the resolution of sexual antagonism in diploid systems. The patterns of sex-biased gene expression in *Ectocarpus* may be explained both by the intrinsic characteristics of UV sexual systems and by the distinctive aspects of this organism's reproductive biology.

INTRODUCTION

In many animal and plant species, males differ markedly from females in morphology, physiology and behaviour. Most of these phenotypic differences are mediated by differential gene expression in the two sexes (Ellegren and Parsch 2007) and this differential gene expression may involve a significant proportion of the genome, as much as 75% in *Drosophila* for example(Assis et al. 2012). These sexually dimorphic patterns of gene expression evolve as a consequence of different selection pressures acting on males and females.

The advent of new generation sequencing has allowed comparative transcriptomic studies of males and females from a range of different species with separate sexes including *Drosophila* (e.g. Perry et al. (2014)), birds (e.g. Pointer et al. (2013), Uebbing et al. (2013)), cichlid fishes (Böhne et al. 2014), guppies

(Sharma et al. 2014), nematodes (Albritton et al. 2014), moths (Smith et al. 2014), the pea aphid (Jaquiéry et al. 2013) and brown algae (Lipinska et al. 2013; Martins et al. 2013). A general theme that has emerged from these studies across diverse species is that a significant proportion of the genes in the genome exhibit sex-biased expression, indicating that the expression of sexual dimorphism is associated with marked genetic reprogramming. In most cases, however, the species studied exhibit a high degree of sexual dimorphism and further analyses of species displaying different degrees of sexual dimorphism would be useful to test the correlation between this character and level of sex-biased gene expression.

Studies such as those listed above are starting to provide a comprehensive overview of sex-biased gene expression in a broad range of species, but the evolutionary causes and consequences underlying the patterns of sex-biased gene expression have been examined in only a small subset of these systems. Most of our knowledge on how sex-biased genes evolve comes from work with *Drosophila* and birds (reviewed in (Parsch and Ellegren 2013)), although some studies have also looked at hermaphrodite species and have provided evidence for sexual selection in these systems (Whittle and Johannesson 2013; Gossmann et al. 2014). Evolutionary analyses have identified several unusual features of sex-biased genes. For example, in gonochoristic/dioecious systems, male-biased genes typically evolve more rapidly at the protein level than female-biased or unbiased genes (e.g. (Zhang et al. 2004; Haerty et al. 2007; Assis et al. 2012); reviewed by Ellegren and Parsch (2007); see also (Mank et al. 2007)). This is believed to result from sex differences in selective pressures on genes; the rapid divergence of male-biased genes resulting from sexual selection due to male–male competition or female choice, natural selection, and/or relaxed purifying selection arising from gene dispensability or reduced functional pleiotropy (Ellegren and Parsch 2007; Mank and Ellegren 2009; Parsch and Ellegren 2013).

The genetic nature of the sexual system can also have an influence, both on the distribution of sex-biased genes in the genome and on their patterns of evolution. In XY sexual systems, for example, X chromosomes spend twice as much time in females as they do in males, favouring the accumulation of female rather than male genes on this chromosome. This phenomenon leads to demasculinisation of the X chromosome (or feminisation of the Z in ZW systems (Kaiser and Ellegren 2006; Arunkumar et al. 2009; Leder et al. 2010; Wright et al. 2012; Jaquiéry et al. 2013)). In addition, beneficial, recessive mutations have a greater probability of fixation when they are X-linked than when they are on an autosome because X is hemizygous in males (Mank et al. 2010; Sackton et al. 2014). As a result, genes located on the X evolve more rapidly, the so-called ‘faster-X’ effect. A similar phenomenon is expected for the Z chromosome in ZW systems.

These latter effects have not yet been investigated in so-called UV sexual systems, commonly found in mosses and many algae, in which sexuality is expressed during the haploid phase of the life cycle (Bachtrog et al. 2011). There are several important differences between UV systems and the more intensely studied XY and ZW systems and these are expected to have consequences for the evolution of sex-biased gene expression. For example, in XY and ZW systems recombination is suppressed only for the Y or W chromosome. The X and Z chromosomes can recombine because they are homozygous in one of the sexes. In contrast, in UV systems neither the U nor the V recombines. Moreover, despite the fact that they do not recombine, U and V chromosomes are expected to degenerate less markedly than Y and W chromosomes because they function in a haploid context where both the U and the V are directly exposed to purifying selection (Bull 1978). Finally, loci on both U and the V chromosomes have half the effective population size of loci on an autosome (all else being equal) whereas this is only the case for the Y and W chromosomes in XY and ZW systems. As far as sex-biased genes are concerned, masculinisation or feminisation of sex chromosomes is not expected in UV systems because of the absence of a sex that carries a homozygous sex chromosome. Similarly, a phenomenon similar to the "faster X" effect is not expected because there is no equivalent of the X chromosome, which recombines but is hemizygous in half of the individuals. Moreover, recent transcriptomic studies from a diverse range of species and tissues (reviewed in (Mank 2013)) suggest that incomplete or imperfect dosage compensation may be responsible for an important proportion of sex-biased gene expression. This type of phenomena is not expected to occur in UV systems because there is no equivalent to the homozygous and heterozygous sexes found in XY and ZW systems and therefore no need for dosage compensation.

On the other hand, other features are anticipated to be shared by both diploid (XY, ZW) and haploid (UV) sex-determination systems. For example, in any sexual system resolution of sexual antagonism is expected to be one of the processes that lead to the emergence of sex-biased gene expression. Theoretical models predict that sex chromosomes should accumulate sexually antagonistic genes in their pseudoautosomal regions (PARs) because even partial linkage to the sex-determining region can be adaptive, allowing alleles to be at least partially restricted to the sex for which they are best adapted (Otto et al. 2011; Charlesworth et al. 2014). This effect is expected not only for the PARs of Y and W chromosomes but also for U and V chromosomes. This accumulation of sexually antagonistic genes might be expected to lead to an enrichment of PARs in sex-biased genes (Charlesworth et al. 2014; Kirkpatrick and Guerrero 2014), although note that there is evidence that the relationship between sexual antagonism and sex-biased gene expression may be quite complex (Innocenti and Morrow 2010; Parsch and Ellegren 2013).

This study focused on sex-biased gene expression in the model brown alga *Ectocarpus*. Brown algae are a group of multicellular photosynthetic organisms that have been evolving independently of both animals and green plants for more than a billion years (Cock, Coelho, et al. 2010). As a group, the brown algae are of considerable interest for investigating the origins and evolution of sexual systems because they have a remarkable variety of levels of sexual dimorphism, reproductive system, types of life cycle and sex chromosome system. *Ectocarpus* is a small, filamentous alga that exhibits limited levels of sexual dimorphism, male and female individuals of the sexual phase of its haploid-diploid life cycle, the gametophyte, are morphologically similar organisms and both produce small flagellated gametes (Luthringer et al. 2014). Sex determination in this organism was recently shown to involve a UV sex chromosome system (Ahmed et al. 2014). In this study the level of sexual dimorphism in *Ectocarpus* was precisely quantified using morphometric methods and RNA-seq was used to characterise sex-biased expression. Several unusual features were noted, compared to previously characterised sexual systems. First, fewer than 12 % of *Ectocarpus* genes exhibited sex-biased expression, consistent with the low level of sexual dimorphism in this species. Second, both male and female sex-biased genes showed accelerated rates of evolution compared with unbiased genes, with male- and female-biased genes evolving at a similar pace. This balanced rate of evolution is also consistent with the low level of sexual dimorphism, which presumably provides limited scope for asymmetric sexual selection. Gene duplication has played a significant role in the generation of sex-biased gene in *Ectocarpus* and the evolution of these genes has been shaped by both positive selection and relaxation of purifying selection. We identified no clear effects of the UV sexual system on the genomic distribution of sex-biased genes but the PAR was found to be enriched in female-biased genes expressed during the mature gametophyte stage.

RESULTS

Ectocarpus exhibits a low level of sexual dimorphism

Sex is determined genetically during the haploid gametophyte generation of the *Ectocarpus* haploid-diploid life cycle (Fig. 1) by a UV sexual system (Ahmed et al. 2014). Meiosis occurs during the sporophyte generation, producing meio-spores, which develop into either male or female gametophytes. The gametophyte generation produces either male or female gametes, depending on its sex, in sexual structures called plurilocular gametangia.

Morphometric analysis showed that male gametophytes were significantly smaller than female gametophytes at fertility but that they produced significantly more reproductive structures (plurilocular gametangia) despite their smaller size (Fig. 2A, Student's t-test, $p<0.0001$). Consequently, male gametophytes presumably produce more gametes than females, because they produce a larger number of plurilocular gametangia per individual.

Ectocarpus gametes have been described as being morphologically isogamous and physiologically anisogamous (Schmid 1993). The physiological anisogamy refers to the behaviour of the two types of gamete during the fertilisation process. The female gametes settle rapidly after release from the plurilocular gametangia, loose their flagella and then produce a pheromone to attract male gametes. Male gametes swim for longer and are attracted to the immobile female gametes by the pheromone. We used flow cytometry to precisely measure male and female gamete size in three different species of *Ectocarpus*. This analysis, based on measurements of more than one thousand gametes, showed that male gametes not only exhibit physiological and behavioural differences compared with female gametes, but they are also slightly, but significantly, smaller (Fig. 2B, Mann Whitney U-test, $p<0.0001$).

Taken together, these analyses identified sexual dimorphisms at both the gametophyte and gamete stages that had not been previously described. *Ectocarpus* therefore clearly exhibits sexual dimorphism, but the differences between males and females are subtle.

Analysis of gene expression during the development of the sexual generation, the gametophyte

Gene expression patterns during sexual differentiation were measured by deep sequencing (RNA-seq) of cDNA from haploid male and female gametophytes of *Ectocarpus* at two different sexual developmental stages: in juvenile immature gametophytes before the formation of the sexual structures (ca. 10 days after meio-spore settlement) and at sexual maturity, when sexual structures were visible (Fig. 1). Correlation between biological replicates of each sex and life cycle stage was strong, with r ranging from 0.91 to 0.99 ($P < 2e^{-16}$).

Counts of expressed genes (RPKM>1) identified 13,102 and 12,660 genes that were expressed at the immature stage (male and female respectively) and 13,941 and 13,663 genes that were expressed at maturity (male and female respectively). This indicates that about 88% of the protein-coding genes in the genome are transcribed during the gametophyte generation (Fig. S1).

Sex-biased gene expression

Fewer than 12% of *Ectocarpus* genes showed sex-biased expression during the gametophyte generation (including both immature and fertile stages). This is considerably less than the numbers identified in previously characterized systems with more marked morphological sexual dimorphism such as *Drosophila* (e.g. (Jiang and Machado 2009)) and birds (Pointer et al. 2013) but coherent with the low level of morphological sexual dimorphism in *Ectocarpus*.

Unexpectedly, the number of genes that were differentially transcribed between males and females was higher during the immature gametophyte stage than at gametophyte fertility (Fig. 3A,B). Male-biased genes were more numerous than female-biased genes at both developmental stages, although the numbers for the most strongly differential genes (FC>10) were comparable for the two sexes (Fig. 3A,B and Table S1). The majority of the sex-biased genes showed significant sex-biased expression in only one of the two developmental stages analysed; only 12% of the male- and 3% of the female-biased genes were differentially expressed in both immature and fertile gametophytes (Fig. S2). Moreover, 3% of the genes that showed male-biased expression in the immature gametophytes were female-specific at maturity. Transitions from female-biased to male-biased were not detected.

To examine the relationship between degree of sex-biased expression and transcript abundance (expression level), the sex-biased genes were grouped according to the fold change difference between male and female samples and mean expression level in males and in females plotted for each group (Fig. S3A). This analysis indicated that when genes exhibited a high degree of female-biased expression, this was predominantly due to down-regulation of these genes in males. This was observed at both immature and fertile gametophyte stages. The results obtained for male-biased gene were more complex. In immature gametophytes, the situation was similar to that observed for the female-biased genes in that a high degree of male-biased expression appeared to be correlated with down regulation in females. In contrast, in mature gametophytes, when genes exhibited a high degree of male-biased expression this appeared to be due to a combination of both decreased expression in females and up regulation in males. We also noted that, on average, female-biased genes were expressed at significantly higher levels than male-biased genes in both fertile and immature gametophytes (Mann Whitney U-test, $p < 2e^{-16}$) (Fig. S3B).

Breadth of expression of sex-biased genes

The breadth of expression of a gene, i.e. the extent to which its expression is limited to specific tissues, is a key determinant of its speed of evolution (Duret and Mouchiroud 2000; Zhang and Li 2004; Slotte et al. 2011). In the moss *Funaria hygrometrica*, which also has a haploid-diploid life cycle, the effect of breadth

of expression was shown to be stronger than the masking effect associated with expression during the diploid phase (Szövényi et al. 2013). In organisms with haploid-diploid life cycles, the breadth of expression of sex-biased genes is restricted because they tend to be preferentially expressed during the haploid phase (sexuality is only expressed during this phase of the life cycle). This restricted pattern of expression is expected to have a significant effect on their evolutionary rates.

When determining the breadth of expression of *Ectocarpus* genes, we integrated this latter type of information to obtain meaningful estimates because this species exhibits only a limited level of tissue differentiation during development. We determined the breadth of expression of the sex-biased genes using the specificity index (τ) (see Materials and Methods) and gene expression data collected both for different tissues (upright filaments versus prostrate tissues during the sporophyte generation, Fig. 1) and for different stages of the life cycle (partheno-sporophyte, immature and fertile gametophyte and gamete stages, Fig. 1). Male and female sex-biased genes had significantly higher τ values compared to unbiased genes, indicating that the former have a greater tendency to be expressed specifically in particular tissues or stages of the life cycle. However, no difference in breadth of expression was observed when the male- and female-biased gene sets were compared with each other (Fig. 4).

Functional analysis of sex biased genes

An analysis of gene ontology (GO) terms associated with the sex-biased genes was carried out using Blast2GO (Conesa and Gotz 2008) to search for enrichment in particular functional groups and to relate gene function to phenotypic sexual dimorphisms. Significant enrichment of specific GO categories was only detected for fertile male gametophyte and immature female gametophyte sex-biased genes. The set of male-biased genes in mature gametophytes was enriched for “microtubule” and “calcium binding-related” processes. These genes may be involved in the production of flagellated gametes inside plurilocular gametangia. Note that the same GO categories were enriched in the set of sex-biased genes expressed in male gametes identified by (Lipinska et al. 2013). The set of female-biased genes in juvenile gametophytes was enriched for “photosynthesis” GO terms, consistent with the more extensive growth phase in the female gametophyte.

A test was also carried out to identify GO terms enriched in the expressed gene sets of the immature compared with the fertile developmental stage of the gametophyte, irrespective of sex. Genes involved in post-translational regulation of gene expression, cellular component biogenesis and photosynthesis were significantly enriched in immature compared with fertile gametophytes (FDR<5%), whereas genes predicted to be involved in signalling, microtubule-based processes and energy metabolism were

significantly enriched in mature compared with immature gametophytes (FDR<5%) (Table S2). The enriched gene GO terms were coherent overall with the transition from vegetative growth to reproductive function, particularly the production of flagellated gametes, between these two stages of development.

Genomic locations of sex-biased genes

An analysis of the genomic distribution of sex-biased genes expressed in fertile gametophytes found that the PAR region of the sex chromosome was enriched in female-biased genes expressed at this stage compared to the rest of the genome (Fig. S5, Chi squared test, $p < 0.01$). Moreover, when RPKM values were used to determine the ratios of transcript abundances in fertile female gametophytes compared with fertile male gametophytes for all the PAR genes, a significant bias towards expression in the female was detected, compared to all the autosomal genes (Kruskal-Wallis, $p < 0.001$) (Fig. 5). These tendencies were not observed for sex-biased genes expressed in immature gametophytes. These observations suggest that the PAR and the autosomes are not evolving under the same selection pressures during the fertile gametophyte stage of the life cycle.

Evidence of a role for gene duplication in resolving sexually antagonistic gene functions

Gene duplication is thought to have played a significant role in the evolution of sex-biased gene expression in *Drosophila* (Connallon and Clark 2011; Wyman et al. 2012). Duplication of a gene can release one or both of the duplicated products from selective constraints allowing the evolution of modified patterns of expression or of new gene functions. Within sexual systems, gene duplication represents a potential means to resolve sexual antagonism. The simplest mechanism would be the generation, after duplication, of one male- and one female-biased gene with male- and female-optimised functions, respectively. Other alternatives are possible, however. For example, it may be sufficient for only one member of a duplicated pair to evolve sex-specific functions to resolve a sexual antagonism. In such cases, gene duplication could help resolve sexual conflict for genes with ontogenetic or pleiotropic constraints by allowing one of the duplicated paralogs to evolve sex-biased expression whilst other maintains a general, sex-independent function (Gallach and Betrán 2011; Wyman et al. 2012). It is also possible that duplication of a gene that is already sex-biased may allow one of the duplicates to evolve an even stronger sex-biased function (Wyman et al. 2012).

The *Ectocarpus* genome contains a total of 879 duplicated gene pairs. Of these, 174 pairs included at least one sex-biased gene. Only three of these 174 pairs included both a male-biased and a female

biased gene. For the three autosomal, duplicated gene pairs, sex-biased expression was detected during the immature gametophyte stage. Comparisons with sequence datasets for other Ectocarpales species identified orthologues for only one of the genes from these three autosomal gene pairs (Esi0002_0006) but this locus did not show any signatures of positive selection. The other sex-biased, duplicated gene pairs included 143 pairs in which only one member of the pair exhibited sex-biased expression and 28 pairs where both members exhibited sex-biased expression, but in the same sex. The 143 duplicated gene pairs in which only one member exhibited sex-biased expression potentially correspond to events where gene duplication has released one member of the gene pair from selective constraints allowing it to evolve a sex-specific function. This hypothesis is supported by the fact that the specificity index (τ) values for the non-sex-biased members of these pairs are significantly lower than those of the sex-biased members (Kruskal-Wallis test with Dunn's post-test, $p<10e^{-8}$) and are not significantly different from values for randomly selected single copy unbiased genes (Fig. 7, Fig. S6).

No evidence has been found for whole genome duplication events having occurred in the lineage leading to *Ectocarpus* (Cock, Sterck, et al. 2010), suggesting that the 879 duplicated gene pairs in the genome of this species arose as a result of small-scale duplication events. When the proportion of the genome corresponding to sex-biased genes is taken into account (1947 of 16262 genes), duplicated gene pairs containing at least one sex-biased gene are overrepresented in the total set of 879 duplicated gene pairs (Chi squared test, $p=1.5e^{-12}$). This overrepresentation was also detected if only male-biased (Chi squared test, $p=8.77e^{-6}$) or only female-biased genes (Chi squared test, $p=2.47e^{-5}$) were considered. The results of these tests suggest that the resolution of sexual conflict was one of the forces driving gene duplication in this genome and support a role for gene duplication in the generation of sex-biased genes in this species.

Sex-biased genes are evolving more rapidly

To test for differences in rates of evolutionary divergence between different categories of sex-biased and unbiased genes, we calculated levels of non-synonymous (dN) and synonymous (dS) substitution using pairwise comparisons with orthologues from the sister species *Ectocarpus fasciculatus*.

The results of this analysis indicated that genes that exhibited sex-biased expression patterns (either male- or female-biased expression) in fertile gametophytes had evolved significantly faster (i.e. had higher dN/dS values) than had unbiased genes (Mann Whitney U-test, $p<0.01$). A similar, but weaker, pattern was observed for genes that were male-biased in immature gametophytes (Mann Whitney U-test, $p<0.01$) but the rates of evolution of female-biased genes identified at this developmental stage were not significantly different from those of unbiased genes (Fig. 6A). Therefore, although the evolution

rates of male and female sex-biased genes were similar overall, differences were detected when the developmental stage at which the genes were expressed was taken into account. These differences suggest not only that the average selection pressure may vary during development, but also that there may be some asymmetry in the evolution rates of these male- and female-biased genes at particular developmental stages. Concerning this latter point, however, it is possible that the stage at which the comparison was carried is not directly comparable in males and females because the immature females delay reproduction in order to prolong growth. The comparison is therefore between a stage in males where there may already have been a cryptic transition towards the reproductive phase, as indicated by the greater overlap between the male-biased gene sets identified in immature and fertile individuals, and a stage in females which is equivalent in terms of timing but which corresponds to a continuation of the pre-reproductive growth phase.

The elevated dN/dS values for sex-biased compared to unbiased genes, were due to significantly higher levels of non-synonymous substitution (Mann Whitney U-test, $p<0.05$) and not to a reduction in the synonymous substitution rate (Fig. 6B). Analysis of the distribution of dN/dS values indicated that the different groups of sex-biased genes (i.e. male- or female-biased, expressed in immature or fertile gametophyte) tended to be enriched in genes with high dN/dS values, including values of one or more, and to contain fewer genes under strong selective constraint (dN/dS <0.1) compared to the group of unbiased genes (Fig. 6C).

Analysis of specificity index (τ) values indicated that the rates of evolution of the sex-biased genes were only weakly correlated with breadth of expression (ρ = 0.1395, p= 0.0229). This suggests that the effect of sex-biased expression on evolution rate was not solely an indirect effect of restricting gene expression patterns.

Expression bias in sexual tissues has been associated with optimal codon usage, a feature that promotes efficient translation (Duret 2000; Duret and Mouchiroud 2000). For instance, optimal codons occur less frequently in male-biased than in female-biased sexual genes in *Drosophila* (Hambuch and Parsch 2005), suggesting that adaptive protein evolution has modified selection on codon usage. Calculations of the Effective Number of Codons (ENC) and the Codon Adaptation Index (CAI) indicated that selection to maintain codon usage bias in *Ectocarpus* sex biased genes is globally preserved (Fig. S4A,B).

As expected, codon usage bias was strongly correlated with the level of gene expression in *Ectocarpus* (CAI vs logRPKM, Spearman's ρ=0.623, p=3.76e^{-06}). A slight decrease in CAI was observed in female-biased compared with unbiased genes (Mann-Whitney test, p=0.02) but there was no significant

difference in codon usage parameters (CAI and ENC) either between the male-biased genes and unbiased genes or between male and female SBGs.

Evidence for positive selection of sex-biased genes

To assess whether differences in divergence rates were due to increased positive selection or relaxed purifying selection, we used sequence data from several Ectocarpales species (Table S3) to estimate direction of selection. We tested 137 sex-biased genes (65 female-biased and 72 male-biased; including 12 genes with dN/dS >0.5) and 40 randomly selected unbiased genes using the paired nested site models (M1a, M2a; M7, M8) implemented in PAML4 (Codeml) (Yang 1998; Yang et al. 2000; Yang 2000). The second model in each pair (M2a and M8) is derived from the first by allowing variable dN/dS ratios between sites to be greater than one, making it possible to detect positive selection at critical amino acid residues. This analysis detected evidence of positive selection for five of the 12 sex-biased genes with dN/dS values of >0.5, including both male- and female-biased genes. Moreover, evidence of positive selection was also found for 12 of the remaining 125 SBGs based on either one or both pairs of models (M1a-M2a, M7-M8) with lower dN/dS values (Table S4). Therefore, the application of the site models of codon evolution indicated that, in contrast to the set of unbiased genes which contained no genes with signatures of adaptive evolution, the set of sex-biased genes was significantly enriched in genes that were under positive selection (Fisher's exact test, p=0.0259).

DISCUSSION

A complex relationship across sexual species between the proportion of the transcriptome showing sex-biased expression and the degree of sexual dimorphism

Analyses of sex-biased gene expression in *Drosophila* have shown that a large proportion of the transcriptome is differentially expressed in the two sexes (Ellegren and Parsch 2007; Jiang and Machado 2009; Assis et al. 2012). A similar observation was made for turkeys, where it was further shown that male-biased gene expression is significantly enhanced, across the genome, in dominant compared with subordinate males (Pointer, et al. 2013). Given that dominant males exhibit stronger secondary sexual characteristics than subordinates, these studies indicate a direct correlation between the degree of sex-biased gene expression and the extent of sexual dimorphism. However, there is also evidence that the relationship between the level of sex-biased gene expression and the degree of sexual dimorphism may be more complicated. For example, in *Drosophila* more sex-biased genes were detected during the

juvenile stage than in adults, despite the lower degree of observable sexual dimorphism during the former phase of development (Mank et al. 2010; Perry et al. 2014). Further studies are therefore required to investigate the exact relationship between these two parameters.

Ectocarpus represents an interesting system in this respect because the studies that have been carried out to date have focused on species that exhibit very marked sexual dimorphism. In contrast, we show here that this brown alga exhibits a limited degree of sexual dimorphism, restricted to subtle growth-habit and fertility differences during the gametophyte stage and a small difference in male and female gamete size. Accordingly, less than 12% of the genes in the genome were found to be differentially regulated between sexes, supporting the hypothesis that the overall degree of sex-biased gene expression and the level of phenotypic sexual dimorphism are correlated.

Analysis of the expression of *Ectocarpus* sex-biased genes during development revealed a more complex relationship between the expression patterns of these genes and the manifestation of sexually dimorphic traits. As observed with *Drosophila*, more sex-biased genes were detected during the sexually immature stage than in fertile, sexually mature individuals, despite the fact that the former exhibited less marked sexual dimorphism. Similarly, male and female gametes have been shown to exhibit high levels of sex-biased expression despite limited phenotypic sexual dimorphism (Lipinska et al. 2013). Thus there is evidence in both *Drosophila* and *Ectocarpus* that the correlation between the level of sex-biased gene expression and the level of observed sexual dimorphism breaks down to some extent when the relationship is examined over the course of development. As *Ectocarpus* and *Drosophila* are two phylogenetically distant organisms with very marked differences in their levels of sexual dimorphism, these observations suggest that the lack of correlation between sex-biased gene expression and sexual dimorphism in immature individuals may be a general feature of sexual systems, but further studies on diverse sexual organisms are required to confirm this. In summary, therefore, whilst there appears to be a broad correlation between the proportion of the transcriptome that exhibits sex-biased expression and the degree of sexual dimorphism, these two phenomena may not be absolutely correlated during the development of the organism.

Analysis of predicted gene functions indicated that about 12% of the male-biased genes expressed during the immature stage were also expressed in fertile gametophytes, but there was less overlap between female-biased genes expressed at the two stages (3% of the female-biased genes). This suggests that immature females were principally carrying out processes unrelated to those engaged at maturity, such as filamentous growth for example, whereas reproductive processes were already

initiated to some extent in immature males, before any phenotypic change could be detected. Somewhat paradoxically, therefore, one of the roles of sex-biased genes in females may be to suspend reproductive functions to allow more extensive vegetative growth during the juvenile phase.

As far as the mechanism of evolution of the sex-biased genes in *Ectocarpus* is concerned, the set of sex-biased genes in this species is enriched in genes that are members of duplicated pairs indicating that neo- or sub-functionalization following gene duplication is one of the mechanisms via which sex-biased genes evolve in this brown alga. Gene duplication has been proposed to be one of the means of resolving sexually antagonistic conflict in other systems (Connallon and Clark 2011; Gallach and Betrán 2011; Wyman et al. 2012).

Symmetrical evolution rates of male- and female-biased genes in *Ectocarpus*

In general, sex-biased genes tend to evolve at faster rates than unbiased genes and this effect is usually significantly more marked for male-biased genes than for female-biased genes (reviewed in (Ellegren and Parsch 2007)). The faster evolution rate is thought to be due, at least in part, to positive selection acting on the sex-biased genes, the most likely underlying causes being sexual selection and/or sexual antagonism. The sex-biased genes in *Ectocarpus* also exhibit faster evolution rates than unbiased genes but this system is unusual in that, overall, male- and female-biased genes have evolved at similar rates. There are several possible explanations for this symmetry. The most obvious explanation, which is consistent with the low level of sexual dimorphism in this system, is that male- and female-biased genes are under similar levels of sexual selection. Both male and female gametes are small, motile cells that are produced in large numbers in plurilocular gametangia by male and female gametophytes, respectively. It is not known whether gamete competition occurs during fertilisation under natural conditions but, if it does occur, the mechanism involved affords scope for both male and female competition. Male gametes may compete to find and fertilise the settled female gametes, but the abundant female gametes may compete for optimal niches in which to settle and then compete with each other to attract male gametes through pheromone production. It is therefore quite possible that selection pressures on males and females are very similar in this organism.

Sex-biased genes in *Ectocarpus* are expressed during the haploid phase of the cycle and therefore directly exposed to purifying selection (Kondrashov and Crow 1991; Orr and Otto 1994; Gerstein et al. 2011). Another possible explanation for the symmetric evolution rates of male- and female-biased genes in *Ectocarpus* may be that haploid phase purifying selection is strong enough to mask any effects of sexual selection or sexual antagonism. This seems unlikely, however, as land plants also possess a

haploid gametophyte generation and selection-driven evolution suggestive of sexual selection has been detected in this group of organisms (Arunkumar et al. 2013; Gossmann et al. 2014).

Another possible factor affecting evolution rate is breadth of expression pattern, as broadly expressed genes tend to be more constrained and therefore to evolve less rapidly than genes with restricted patterns of expression (Hastings 1996; Duret and Mouchiroud 2000). In *Drosophila* one of the reasons that female-biased genes evolve less quickly than male-biased genes may be that, in general, they tend to have broader patterns of expression (e.g.(Meisel 2011; Grath and Parsch 2012)). Our analysis, based on RNA-seq analysis of multiple life cycle stages and tissues, indicated that, in contrast, both male- and female-biased genes in *Ectocarpus* tend to have restricted patterns of expression compared with unbiased genes (Fig. 4). This parallel reduction in breadth of expression may be one of the factors underlying the symmetrical accelerated evolution of male- and female-biased genes in this species. However, we noted that there was only a weak positive correlation between expression breadth (τ) and evolutionary rate (dN/dS), suggesting that other factors have also influenced evolutionary rates.

In summary, therefore, possible explanations for the symmetrical rates of evolution of male- and female-biased genes in *Ectocarpus* include limited sexual selection due to a low level of sexual dimorphism and comparable levels of breadth of expression pattern.

Sexual selection is one of the forces that drives the evolution of male- and female-biased genes in *Ectocarpus*

The mean dN/dS value for sex-biased genes in *Ectocarpus* was more than twice as high as that of unbiased genes. This difference, which was particularly marked for genes expressed in fertile gametophytes, was due to a significantly higher rate of non-synonymous changes compared with the unbiased genes. A test for adaptive evolution detected evidence for positive selection in a significant proportion of the sex-biased genes with the highest dN/dS values (>0.5). Similar observations have been made for sperm-specific genes in *Arabidopsis thaliana* (Arunkumar, et al. 2013) and for gametophyte-specific genes in moss *Funaria hygrometrica* (Szovenyi, et al. 2013). The evidence that positive selection acts on a considerable number of *Ectocarpus* sex-biased genes indicates that sexual selection may be one of the forces driving their evolution. Note however that positive selection only affects a subset of the *Ectocarpus* sex-biased genes and a significant proportion appear to be under relaxed selection. One important consideration in this respect is that a gene that is expressed in only one sex will experience half as much purifying selection because selection can only act on the gene when it is in the appropriate sex (Barker et al. 2005).

Patterns of genomic distribution of sex-biased genes

In XY and ZW systems, the pattern of segregation of the sex chromosomes can have a measurable influence on the distributions of sex-biased genes on this linkage group. For XY systems, for example, X chromosomes spend twice as much time in females as they do in males and this leads to demasculinisation of (i.e. loss of male-biased genes from) the X chromosome (Bachtrog et al. 2010; Leder et al. 2010). There is no equivalent to this phenomenon in UV systems because the sex chromosomes function in the haploid generation. However, UV systems may share other features with XY and ZW systems that affect the distribution of sex-biased genes. In particular, even partial linkage to the sex-determining region can be beneficial for genes with sexually antagonistic alleles, allowing alleles to segregate preferentially to the sex for which they are most adaptive (Otto et al. 2011; Jordan and Charlesworth 2012). This is predicted to lead to the accumulation of sexually antagonistic genes in the PAR, which in turn could lead to an accumulation of sex-biased genes in this region because sex-biased expression is one of the possible mechanisms of resolving sexual antagonism. There is some experimental evidence for this mechanism from work on the ZW sexual system of the emu, which has shown that the PARs of the homomorphic sex chromosomes of this species are enriched in male-biased genes (Vicoso et al. 2013). As expected, this effect was most pronounced for genes expressed in older embryos with fully developed gonads.

For UV systems, in the absence of any additional selective pressure favouring genes of one sex or the other, this effect of linkage to the SDR would not be expected to lead to a preferential accumulation of male-biased genes compared to female-biased genes or vice versa, but it might be expected to result in a general excess of sex-biased genes in the PAR. We did not observe any such excess in *Ectocarpus*, the proportion of sex-biased genes in the PAR was not significantly different to the proportion in the autosomes. However, compared to the autosomes, the *Ectocarpus* PAR was found to be significantly enriched in genes that exhibited female bias expression during the fertile gametophyte stage. One possible explanation for this enrichment in female-biased genes may be a combination of an effect of linkage to the SDR together with stronger selection for female-biased genes during the fertile gametophyte stage.

There is accumulating evidence that gene duplication has played a significant role in the evolution of sex-biased genes in animals (Connallon and Clark 2011; Gallach and Betrán 2011; Wyman et al. 2012) and the data presented here indicates that this has also been the case for *Ectocarpus*, suggesting that similar mechanisms may be operating to generate sex-biased genes across diverse eukaryote sexual systems.

METHODS

Biological material

Ectocarpus strains were cultured at 13°C in autoclaved natural sea water (NSW) supplemented with half-strength Provasoli solution (PES; (Starr and Zeikus 1993)) with a light:dark cycle of 12h:12 (20 µmol photons m^{-2} s^{-1}) using daylight-type fluorescent tubes. All manipulations were performed under a laminar flow hood in sterile conditions. Near-isogenic lines, Ec602 female and Ec603 male, were prepared by crossing brothers and sisters for 8 generations. This produced male and female strains with essentially identical genetic backgrounds apart for the sex locus.

Male and female gametophytes of *Scytosiphon lomentaria* were collected in Asari, Japan in March 2012. *Scytosiphon lomentaria* was cultured in NSW with full strength PES. Two different light conditions were required to complete the life cycle. Short-day conditions, with a light:dark cycle of 10:14h (20 µmol photons m^{-} s^{-s}), were used to produce unilocular sporangia from a diploid sporophyte. After a month approximately 100 young gametophytes were isolated. The gametophytes were then subjected to long-day conditions with a cycle of 14:10h to induce gametophyte maturation. Gametophytes became fertile after approximately four weeks and were frozen in liquid nitrogen. Each individual was sexed by crossing with male and female tester lines.

Measurement of gamete size

Male and female gamete size was measured in three different *Ectocarpus* species (see (Stache-Crain et al. 1997) for a description of the lineage structure of the genus *Ectocarpus*): isogenic male and female strains of *Ectocarpus* sp. from Peru (Ec602 and Ec603; lineage 1c), *E. siliculosus* from Naples (lineage 1a) and *Ectocarpus* sp. from New Zealand (lineage 4). Synchronous release of gametes from 3-4 week old cultures was induced by transferring ten gametophytes to a humid chamber in the dark for approximately 14 hours at 13°C followed by the addition of fresh PES-supplemented NSW medium under strong light irradiation. Gametes were concentrated by phototaxis using unidirectional light, and collected in Eppendorf tubes. Gamete size was measure by impedance-based flow cytometry (Cell Lab QuantaTM SC MPL, Beckman Coulter®). Values of gamete size shown represent the mean ±s.e. of each gamete and measurements were taken for at least three biological replicates. A t-test (α=5%) was performed using GraphPad Prism software to compare female and male gamete size.

Measurement of gametophyte size and fertility

For the analysis of gametophyte habit and fertility, male and female near-isogenic strains (Ec602 and Ec603; Table S3) were placed in culture conditions as described above at constant density (10 individuals per 140 mm Petri dish). In each Petri dish, all 10 gametophytes grew synchronously and attained approximately the same size. The gametophytes attained sexual maturity (production of plurilocular gametangia) after 3-4 weeks in culture. The number of plurilocular gametangia, each containing approximately 300 gametes, was counted under an inverted microscope for one individual randomly taken from each Petri dish. It was not possible to accurately weight a single gametophyte, so ten gametophytes were pooled, weighed and the individual weight estimated by dividing by ten. Results shown correspond to the mean ±s.e. for 6 biological replicates for Ec602 and 8 biological replicates for Ec603. Significant differences were tested using a corrected t-test with R software (α=5%).

RNA extraction

RNA-seq analysis was carried out to compare the relative abundances of gene transcripts at different developmental stages of the life cycle (Fig. 1). For the gametophyte stage tissue preparation, synchronous cultures of gametophytes of the near-isogenic male and female lines Ec603 and Ec602 were prepared under standard conditions (Coelho et al. 2012) and frozen at early stages of development (ca. ten days after release of the meio-spores) and at fertility (presence of plurilocular gametangia). Total RNA was extracted from 2 bulks of 400 male individuals and 2 bulks of 400 female individuals (2 biological replicates for each sex) using the Qiagen Mini kit (http://www.qiagen.com) as previously described (Coelho, et al. 2012). Two biological replicates of basal partheno-sporophyte filaments from strain Ec32 were frozen in liquid nitrogen ten days after settlement of gametes. Similarly, two biological replicates of upright filament tissue were isolated 15 days after settlement of gametes.

Two biological replicates for each sex of *Scytosiphon lomentaria* were prepared by pooling between 8 and 12 individuals per sample. RNA from male and female pools was extracted using the protocol described by Apt et al. (1995). RNA quality and quantity was assessed using an Agilent 2100 bioanalyzer, associated with an RNA 6000 Nano kit.

RNA-seq

For each replicate, the RNA was quantified and cDNA was synthesised using an oligo-dT primer. The cDNA was fragmented, cloned, and sequenced by Fasteris (CH-1228 Plan-les-Ouates, Switzerland). Table S5 shows the statistics for the sequencing and mapping. Data quality was assessed using FASTX toolkit and the reads were trimmed and filtered using a quality threshold of 25 (base calling) and a minimal size

of 60bp. Only reads in which more than 75% of nucleotides had a minimal quality threshold of 20 were retained.

Filtered reads were mapped to the *Ectocarpus* sp. genome (Cock, Sterck, et al. 2010)(available at ORCAE (Sterck et al. 2012) using TopHat2 with the Bowtie2 aligner (Kim et al. 2013: 2). More than 90% of the sequencing reads for each library could be mapped sequences to the genome. The mapped sequencing data was then processed with HTSeq (Anders et al. 2014) to obtain counts for sequencing reads mapped to exons. Expression values were represented as RPKM (reads per kilobase per million mapped sequence reads) and a filter of RPKM>1 was applied to remove noise and genes with very low expression levels (Fig. S1). This resulted in a total of 14,302 genes with expression values above the threshold.

Differential expression analysis was performed with the DESeq package (Bioconductor) (Anders and Huber 2010) using an adjusted p-value cut-off of 0.1 and a minimal fold-change of 2 (Fig. S2). Full lists of SBGs can be found in Table S1.

The sex-biased genes were also analysed for the presence of duplicated genes, to determine whether tandem duplications might have arisen to resolve sexual conflict. The clustering analysis was performed using the MCL algorithm (Markov Cluster Algorithm) with the inflation value fixed to 3.0 and Blastp with a minimal E-value set to $1e10^{-4}$.

Measurement of synonymous and non-synonymous mutation rates

To estimate evolutionary rates of sex-biased genes we searched *E. fasciculatus* transcriptome data (Gachon et al, unpublished) for orthologues of sex-biased and unbiased control genes (the latter was a random subset of 47 genes without differences in expression levels between males and females) by retaining best reciprocal Blastn matches with a minimum e-value of $10e^{-}10$. The orthology of genes derived from duplications in *Ectocarpus* sp. was further evaluated by calculation of phylogenetic trees using *E. siliculosus* and *E. fasciculatus,* along with *S. lomentaria* as an outgroup. MEGA6 (Larkin et al. 2007; Tamura et al. 2013) was used for maximum likelihood analyses and branch support was assessed with by bootstrapping (1000 replicates).

Putative orthologues were aligned using ClustalW implemented in Mega6 (Larkin et al. 2007; Tamura et al. 2013) and manually curated. Sequences that produced a gapless alignment that exceeded 100bp were retained for pairwise dN/dS (ω) analysis using Phylogenetic Analysis by Maximum Likelihood (PAML, codeml, F3x4 model, runmode=-2) implemented in the PAL2NAL suit (Suyama et al. 2006: 2; Yang

2007). Genes with saturated synonymous substitution values (dS>1) and genes located in the sex-determining region were excluded from the analysis.

The Effective Number of Codons (ENC) and the Codon Adaptation Index (CAI) were calculated for all sex-biased and unbiased genes in this study using CAIcal server (http://genomes.urv.es/CAIcal/) (Puigbò et al. 2008).

Positive selection analysis

We used transcriptomic and genomic data from four different *Ectocarpus* species and another Ectocarpales species, *S. lomentaria* to detect positive selection (Table S3). *Ectocarpus* sp. (clade 1c Greenland) and *E. fasciculatus* transcriptome data were generated using using Illumina Hiseq v3 paired-end technology and quality filtered (C. Gachon et al, unpublished). Transcriptome assemblies were generated using the Trinity de-novo assembler (Grabherr et al. 2011) and filtering for isoform percentage (>1) and FPKM (>1).

Genomic data of *E. siliculosus* lineage 1a were aligned to the reference genome and consensus sequences of coding regions with at least 10x coverage were recovered using CLC Assembly Cell (www.clcbio.com).

Orthologues of sex-biased genes which could be aligned over at least 100 bp were identified using a best reciprocal Blastn approach (E-value cutoff of $10e^{-10}$). Nucleotide alignments for genes identified from at least four of the five species were made using CLUSTALW (Larkin et al. 2007; Tamura et al. 2013) implemented in MEGA6 (Tamura et al. 2011), curated manually when necessary and transformed to the PAML4 required format using perl fasta manipulation scripts (provided by Naoki Takebayashi, University Alaska Fairbanks).

Levels of nonsynonymous (dN) and synonymous (dS) substitution were estimated by the maximum likelihood method available in CODEML program (PAML4 package) using the F3X4 model of codon frequencies and a user tree specified according to the phylogeny (Stache-Crain et al. 1997). CODEML paired nested site models (M0, M3; M1a, M2a; M7, M8) (Yang 1998; Yang et al. 2000; Yang 2000)of sequence evolution were used in this analysis and compared using the likelihood ratio test (LRT). Empirical Bayes methods allowed for identification of positively selected sites a posteriori (Yang et al. 2000; Yang 2007).

Breadth of gene expression

RNA-seq data corresponding to complete organisms from seven different stages of the life cycle (male and female gametes, partheno-sporophytes, immature and fertile male and female gametophytes) and to two different tissue types (basal structures and upright filaments) were used to estimate breadth of gene expression. The gamete transcriptomic data (Lipinska et al. 2013) was converted to RPKM in order to make it comparable with other libraries. The tissue specificity index (τ) (Yanai et al. 2005) was used as a measure of breadth of expression for each gene, using the following formula:

$$\tau = \frac{\sum_{i=1}^{N}(1 - x_i)}{N - 1},$$

For each gene we calculated x_i as the expression profile in the given library *i* normalized by the maximal expression value across all analyzed tissues (N). τ index values range from 0 to 1, where 1 corresponds to strong tissue specificity (low expression breadth).

Analysis of predicted gene functions

InterProScan (Zdobnov and Apweiler 2001) and Blast2GO (Conesa and Gotz 2008) were used to recover functional annotations for *Ectocarpus* proteins. A Fisher exact test with an FDR corrected p-value cutoff of 0.05 (Blast2GO) was used to detect enrichment of specific GO-terms in various groups of sex-biased genes.

Genomic location of sex-biased genes

A Chi squared test of observed and expected distribution of SBGs across the *Ectocarpus* linkage groups (Heesch et al. 2010) was used to test whether sex-biased genes were randomly distributed throughout the genome. The expected distribution was calculated with the assumption that the SBGs were randomly distributed and therefore that representation on a particular chromosome should have been proportional to the number of genes on that chromosome. The Chi squared test was performed in Excel 2010 (Microsoft, Redmond, Washington, USA). All other statistical analyses were performed in RStudio (R version 3.0.2) (Anon 2013).

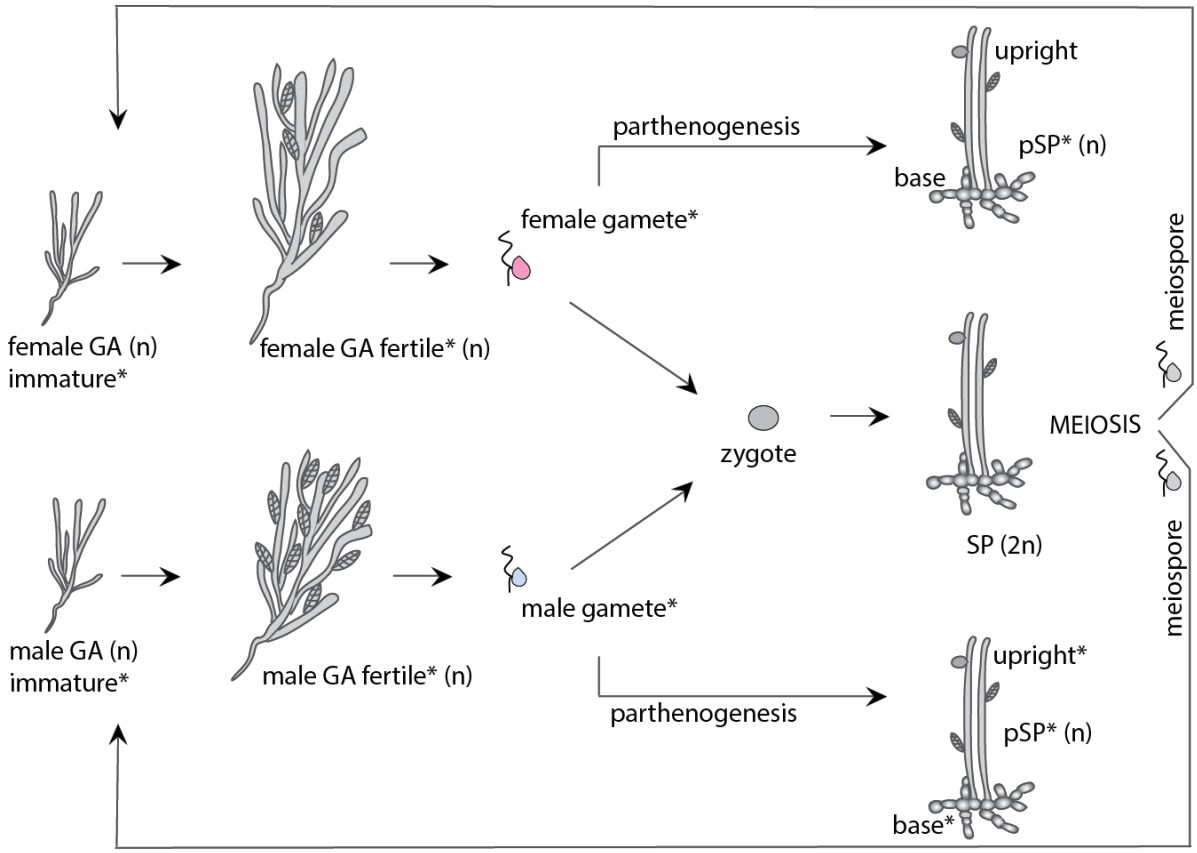
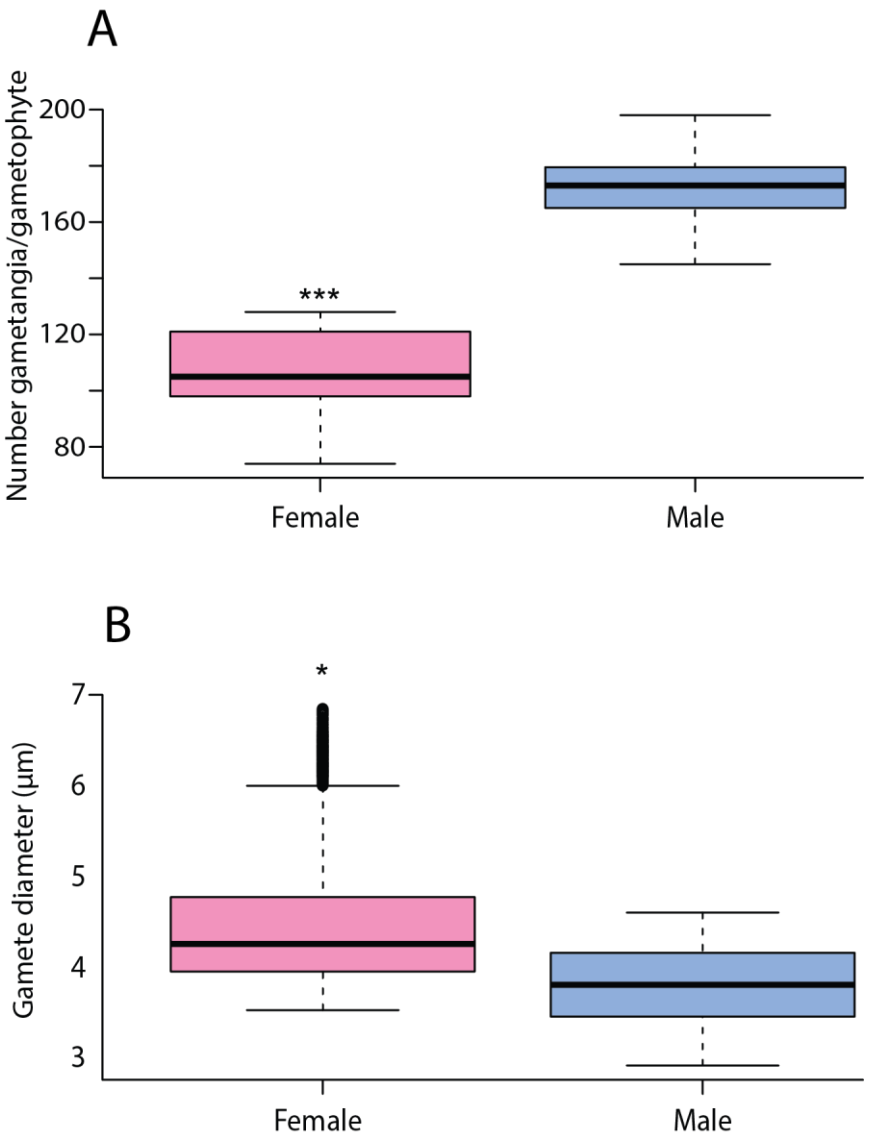


Figure 1. Lipinska *et al.*2014



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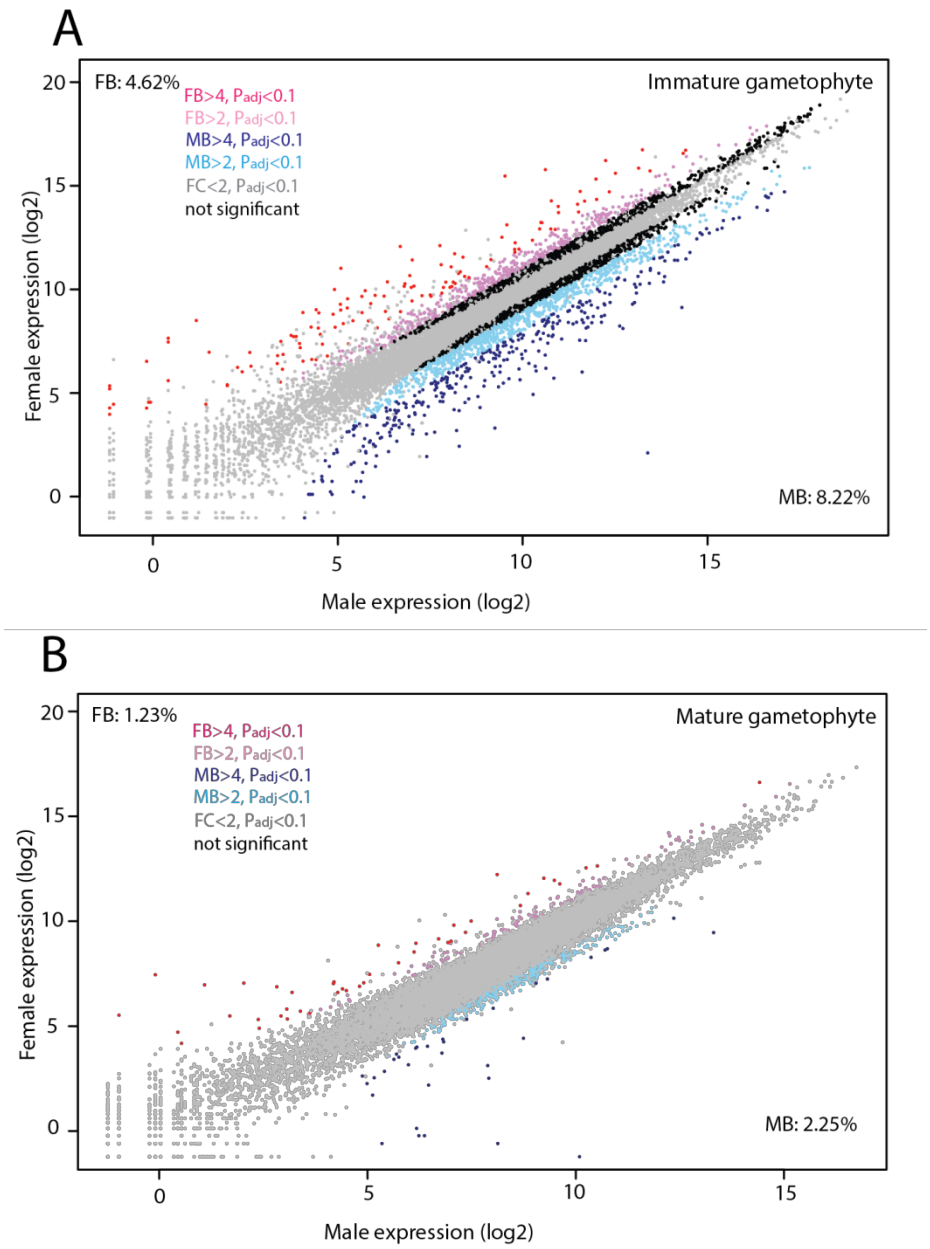
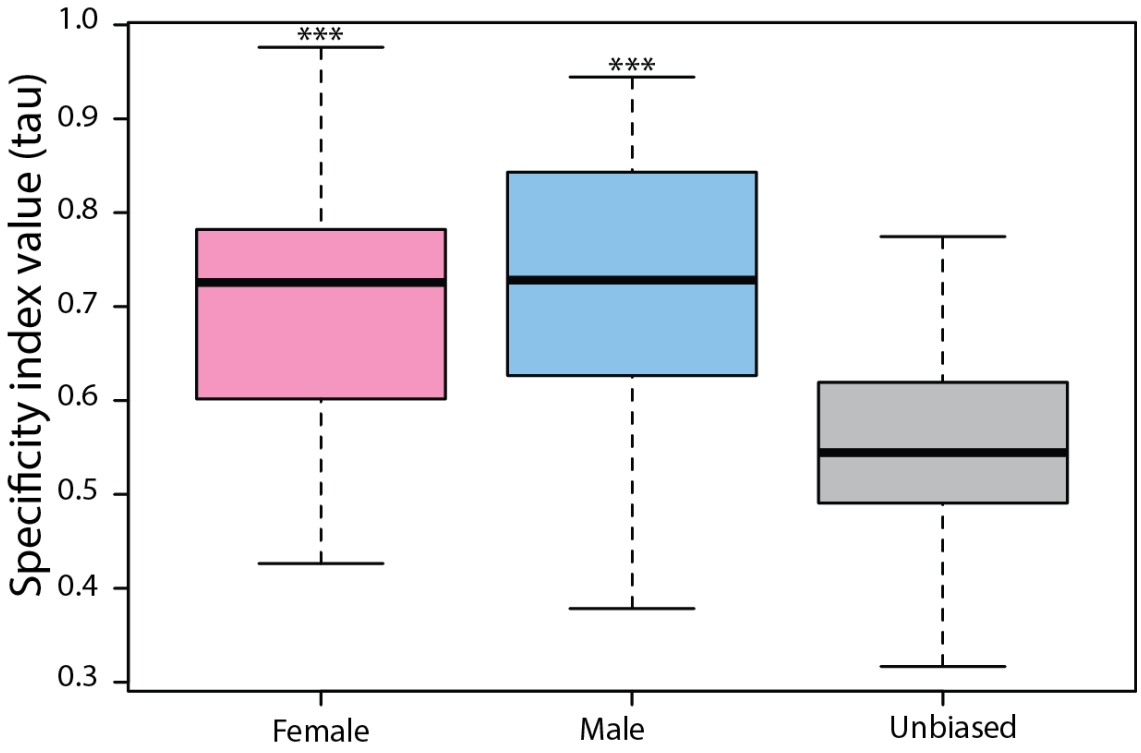


Figure 3. Lipinska *et al.*2014



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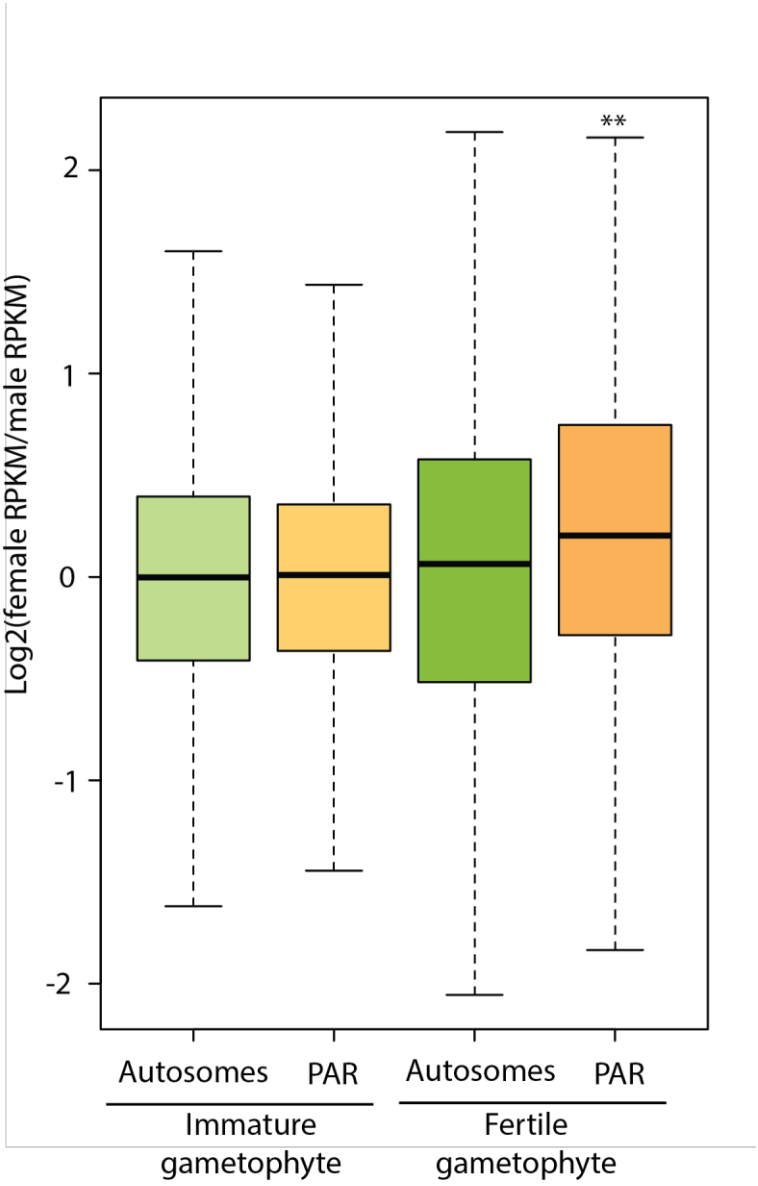

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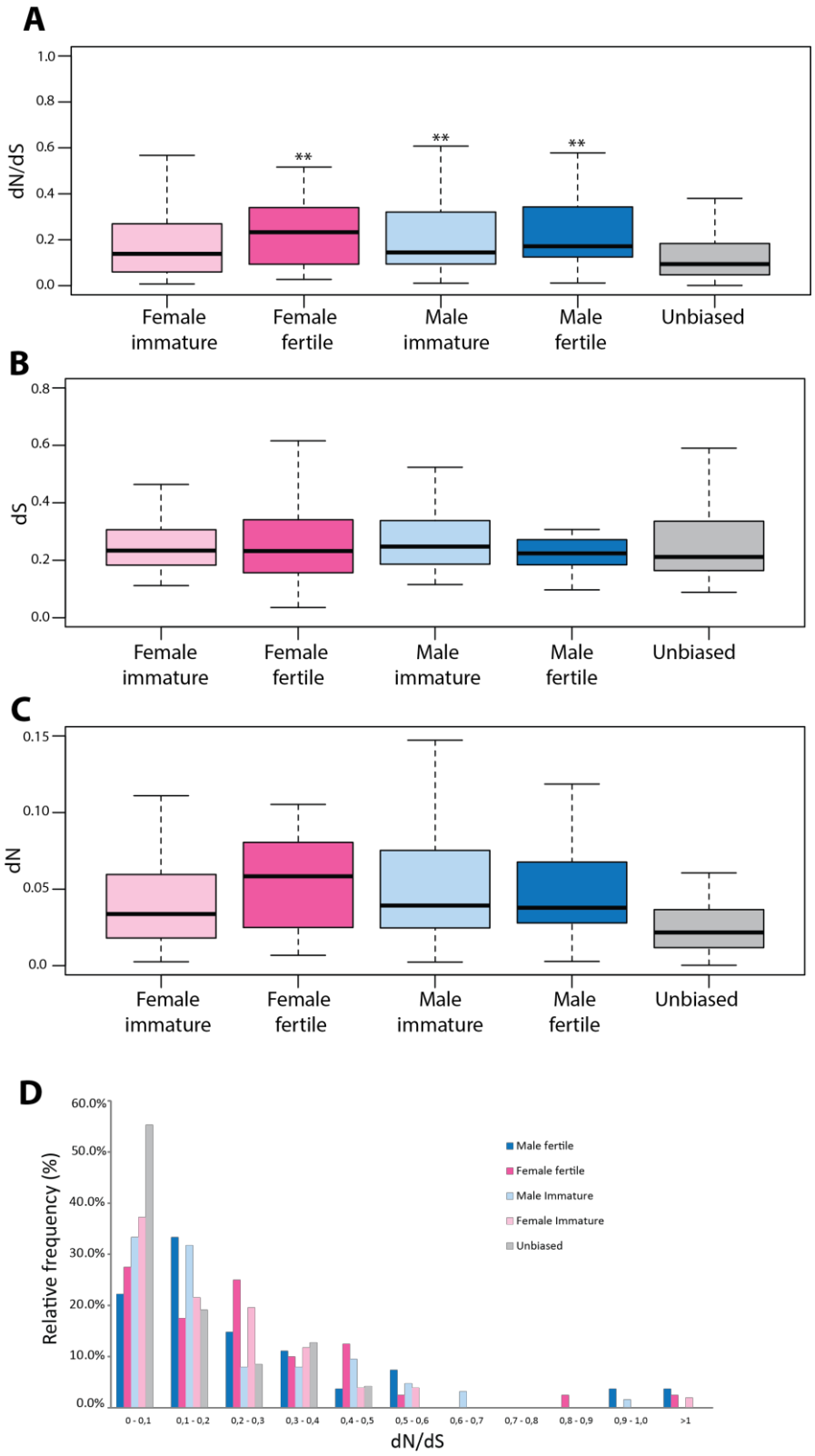

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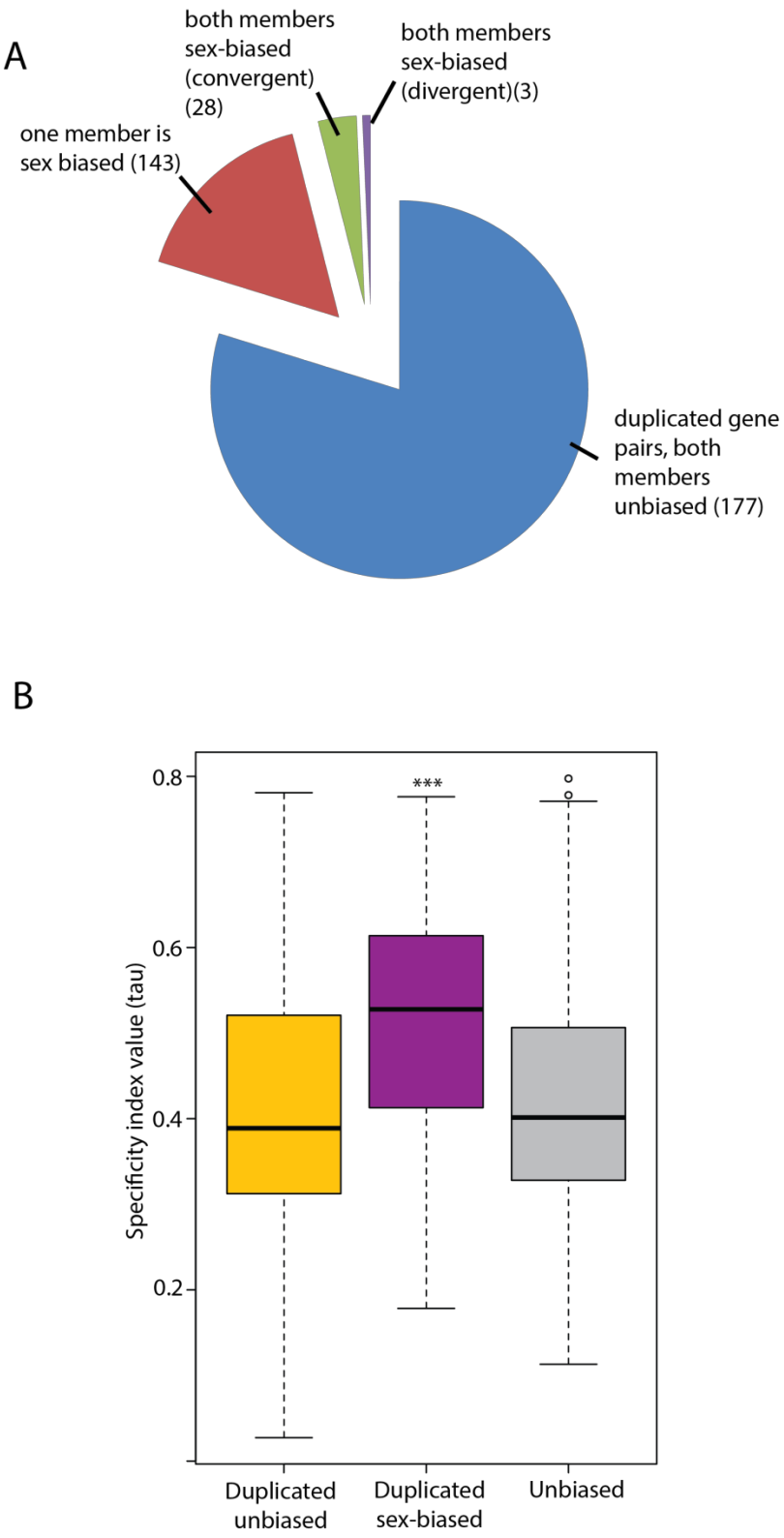

Figure 7. Lipinska *et al.*2014

FIGURE LEGENDS

Figure 1. **The *Ectocarpus* life cycle***.*** The life cycle of *Ectocarpus* sp. involves alternation between two independent multicellular generations, the gametophyte (GA) and the sporophyte (SP). Sporophytes produce meiotic spores (meiospores) that develop into haploid gametophytes, which are either male or female (dioicous). After approximately 3 weeks, gametophytes become fertile and produce gametes in reproductive structures (plurilocular gametangia). After release into the water column, male and female gametes strongly differ in their behaviour and physiology. Female gametes settle rapidly and release a pheromone to attract male gametes, which then fuse with the female gametes to form zygotes (syngamy). Zygotes develop to produce diploid sporophytes, completing the cycle. Gametes that fail to fuse are able to develop parthenogenetically into a haploid partheno-sporophyte (pSP). Parthenogenesis is depicted for both male and female gametes. This is observed in some strains but in the majority of *Ectocarpus* species only the females are capable of parthenogenesis. Partheno-sporophytes are morphologically and functionally indistinguishable from diploid sporophytes. Life cycle stages used for transcriptomic analysis are marked with an asterisk.

Figure 2. Sexual dimorphism in *Ectocarpus* gametophytes. **A)** Number of reproductive structures (plurilocular gametangia) per female (n=6) and male (n=8) gametophyte. Males produced significantly more reproductive structures (Student's t-test, $p<0.0001$). Error bars show standard errors. The number of plurilocular gametangia for each female gametophyte was 128; 109; 74; 121; 101; 98 and for each male gametophyte 176; 145; 198; 178; 169; 170; 181; 161. **B)** Mean diameters (µm) of female (n=5668) and male (n=5619) gametes. Female gametes (mean diameter 4.46µm) were significantly larger (Mann Whitney U-test, $p<0.0001$) than male gametes (mean diameter 3.83µm). Error bars show standard errors. Mean gamete sizes for male and female individuals of other *Ectocarpus* species are provided in the supplementary section.

Figure 3. **Sex-biased gene expression. A**) Comparison of gene expression levels in male and female immature gametophytes. Coloured dots indicate genes that exhibited significantly different levels of transcript abundance (sex-biased genes). Percentages in each panel indicate genes that were at least 2-fold female-biased (FB; upper left) and male-biased (MB; lower right). Fold Change (FC); Female Biased (FB); Male Biased (MB); p adjusted (p_{adj}). Unbiased (UB) genes were defined as $p_{adj}>0.1$ or less than 2-fold difference between the sexes. See also Table 1. **B**) Comparison of gene expression levels in male and female mature gametophytes. Coloured dots indicate genes that exhibited significantly different levels of transcript abundance (sex-biased genes). Percentages in each panel indicate genes that were at least 2-

fold female-biased (FB; upper left) and male-biased (MB; lower right). Fold Change (FC); Female Biased (FB); Male Biased (MB); p adjusted (p_{adj}). Unbiased (UB) genes were defined as p_{adj}>0.1 or less than 2-fold difference between the sexes. See also Table 1.

Figure 4. Breadth of expression of the sex-biased genes as determined using the specificity index. Comparison of specificity index values (τ) for unbiased and sex-biased genes (SBGs). Male and female-biased genes had significantly specificity index values (i.e. lower breadth of expression) compared with unbiased genes (Kruskal-Wallis test, $p < 10^{-5}$).

Figure 5. Comparison of female-to-male expression level ratios for genes on autosomes with genes in the PAR. The figure shows log2 of female/male RPKM ratios for autosomal and PAR genes during the immature and fertile gametophyte stages**.** Outliers were removed from the plot.

Figure 6. Rates of evolution of female-biased, male-biased and unbiased genes. Pairwise dN, dS and dN/dS ratios were calculated by comparing orthologous gene sequences from *Ectocarpus* sp. (clade 1c) and *Ectocarpus fasciculatus*. **A**) Ratio of non-synonymous to synonymous substitutions (dN/dS). **B)** and **C**) Non-synonymous substitutions (dN) and synonymous substitutions (dS). **D**) Frequency of classes of dN/dS ratio in unbiased genes and male- and female-biased genes expressed in immature and fertile gametophytes. Outliers were removed from the plot.

Figure 7. Sex biased gene expression and gene duplication in *Ectocarpus*. A) Distribution of sex-biased genes among the duplicated gene pairs. **B)** Breadth of expression of duplicated gene pairs with one gene being unbiased (Duplicated unbiased) and the second paralog being sex biased (Duplicated sex-biased) as determined using the stage-specificity index (τ). Random unbiased single copy genes (Unbiased) are included for comparison. The median for unbiased members of duplicated pairs is significantly lower than for sex-biased paralogs (Kruskal-Wallis test with Dunn's post-test, $p<10e^{-8}$) and is not significantly different from single copy unbiased genes.

TABLES

Table 1. Relative gene expression for male and female gametophytes.

A) Categories of immature gametophyte sex-biased genes with different levels of fold change (FC) between the two sexes indicated both as number of genes (N° genes) and as a percentage of the total

number of genes expressed (% of expressed genes) in the immature gametophyte of the corresponding sex.

		N° genes	% of expressed genes
Female-biased (p_{adj}<0.1)	FC>2	585	**4.62%**
	FC>4	131	1.03%
	FC>10	68	0.54%
	Total expressed genes (RPKM>1)	12661	
Male-biased (p_{adj}<0.1)	FC>2	1077	**8.22%**
	FC>4	295	2.25%
	FC>10	78	0.60%
	Total expressed genes (RPKM>1)	13102	

B) Categories of fertile gametophyte sex-biased genes with different levels of fold change (FC) between the two sexes indicated both as number of genes (N° genes) and as a percentage of the total number of genes expressed (% of expressed genes) in the mature gametophyte of the corresponding sex.

		N. genes	% expressed genes
Female-biased (p_{adj}<0.1)	FC>2	168	**1.23%**
	FC>4	61	0.45%
	FC>10	29	0.21%
	Total expressed genes (RPKM>1)	13660	
Male-biased (p_{adj}<0.1)	FC>2	314	**2.25%**
	FC>4	54	0.39%
	FC>10	32	0.23%
	Total expressed genes (RPKM>1)	13937	

ACKNOWLEDGEMENTS

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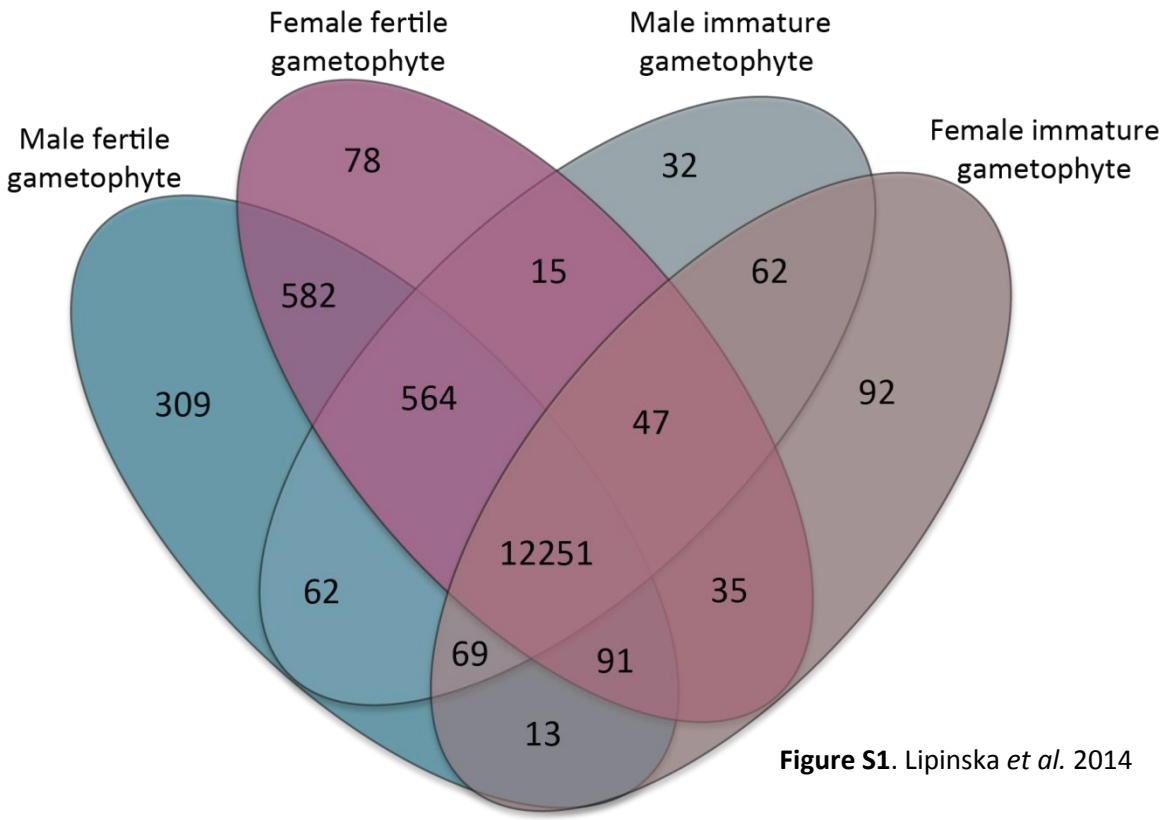


Figure S1. Lipinska *et al.* 2014

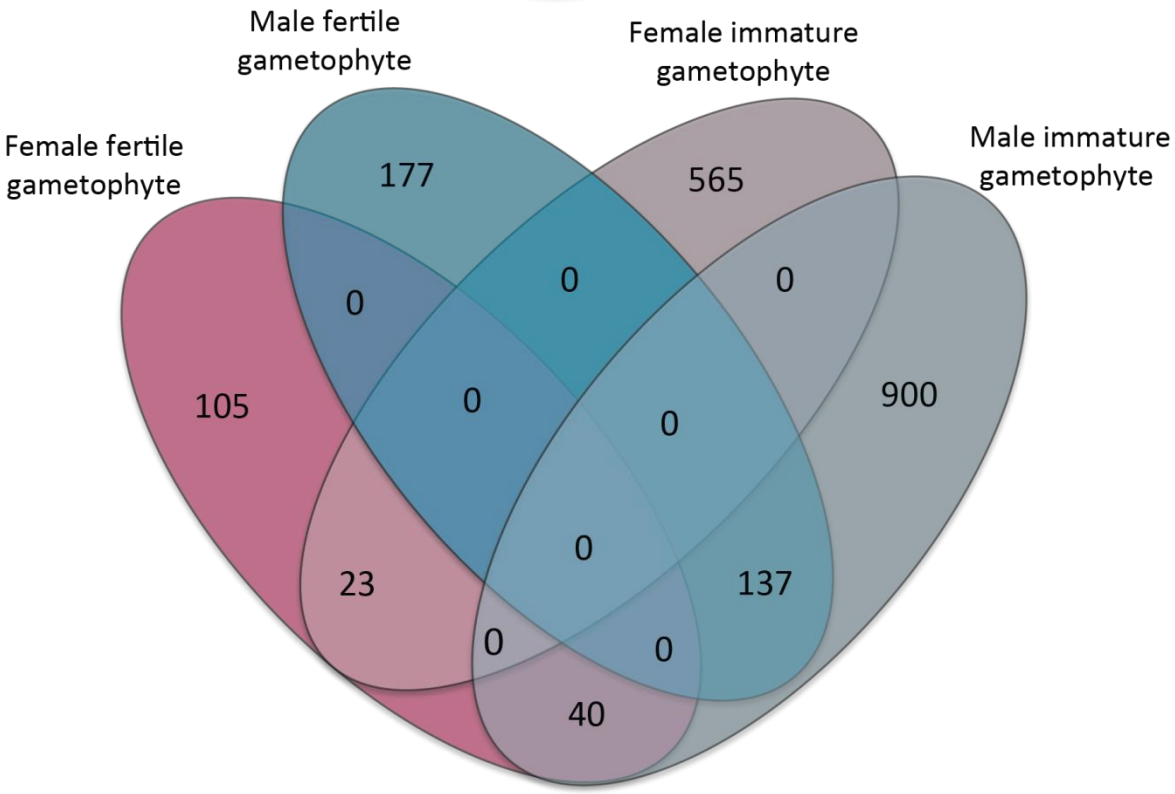
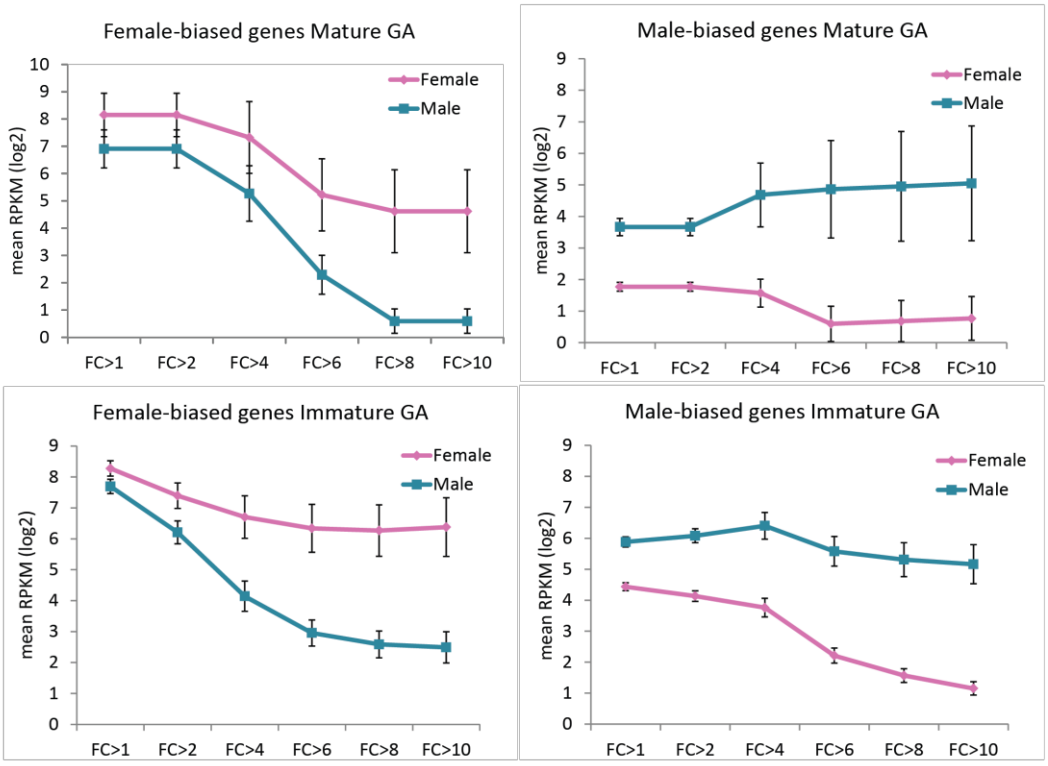


Figure S2. Lipinska *et al.* 2014

A



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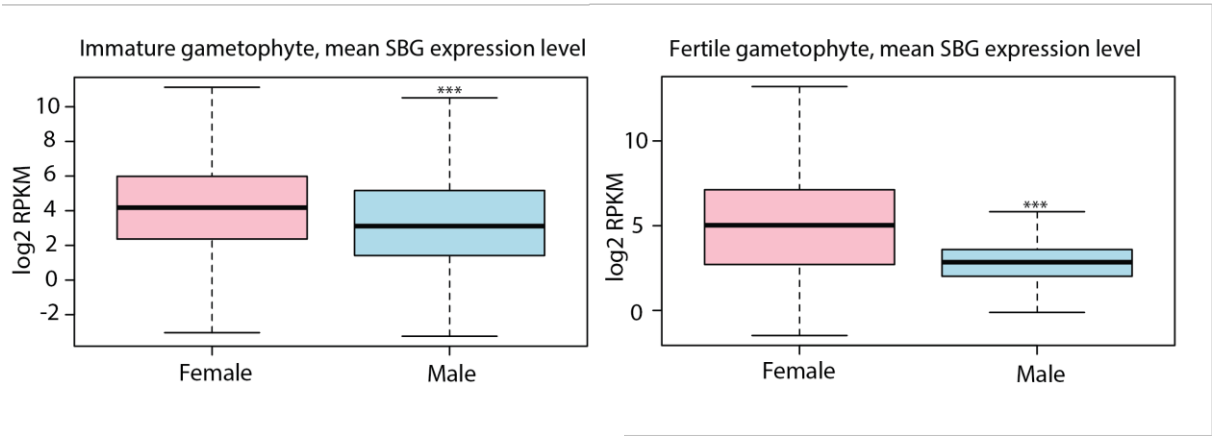

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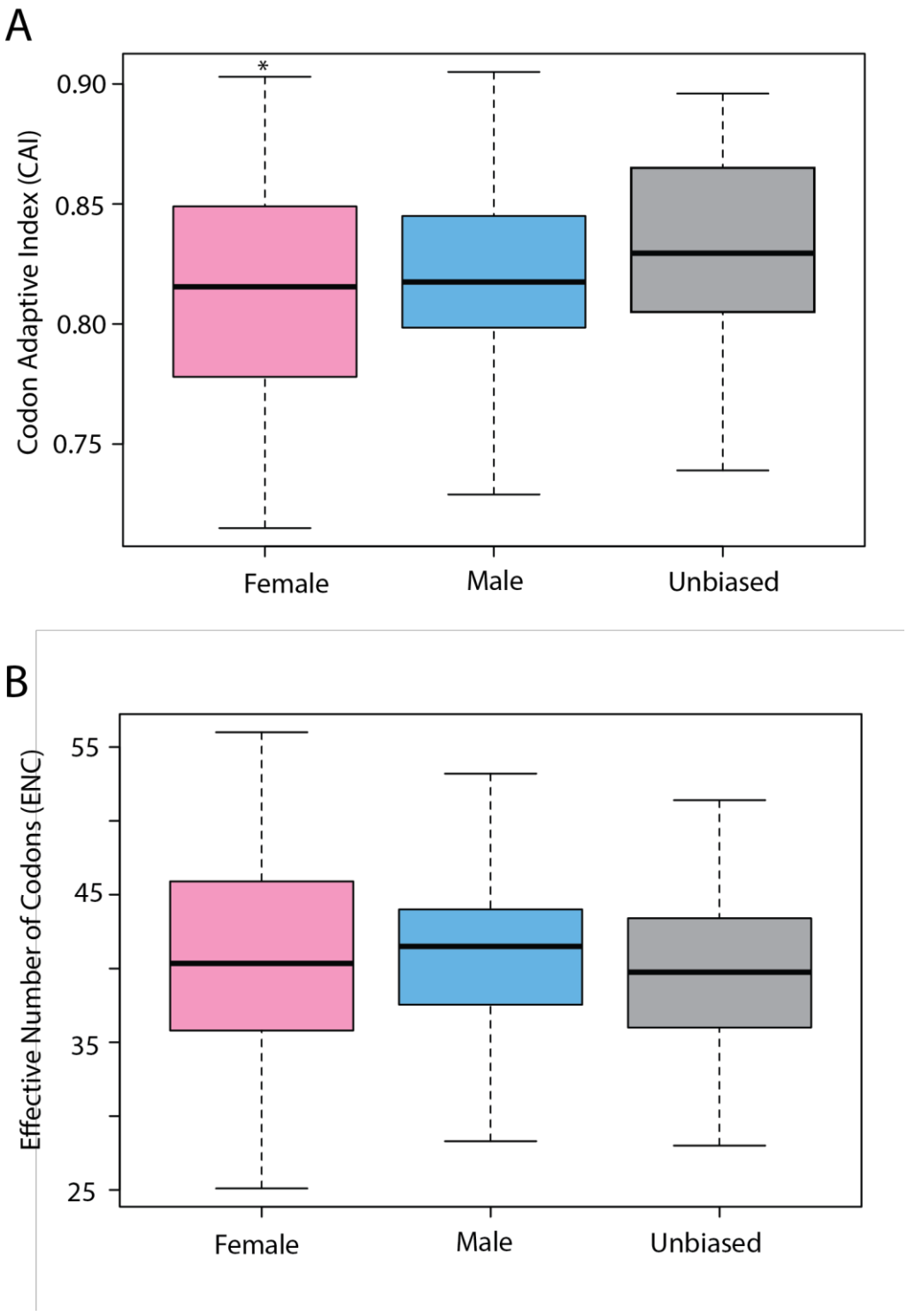

Figure S4. Lipinska *et al.* 2014

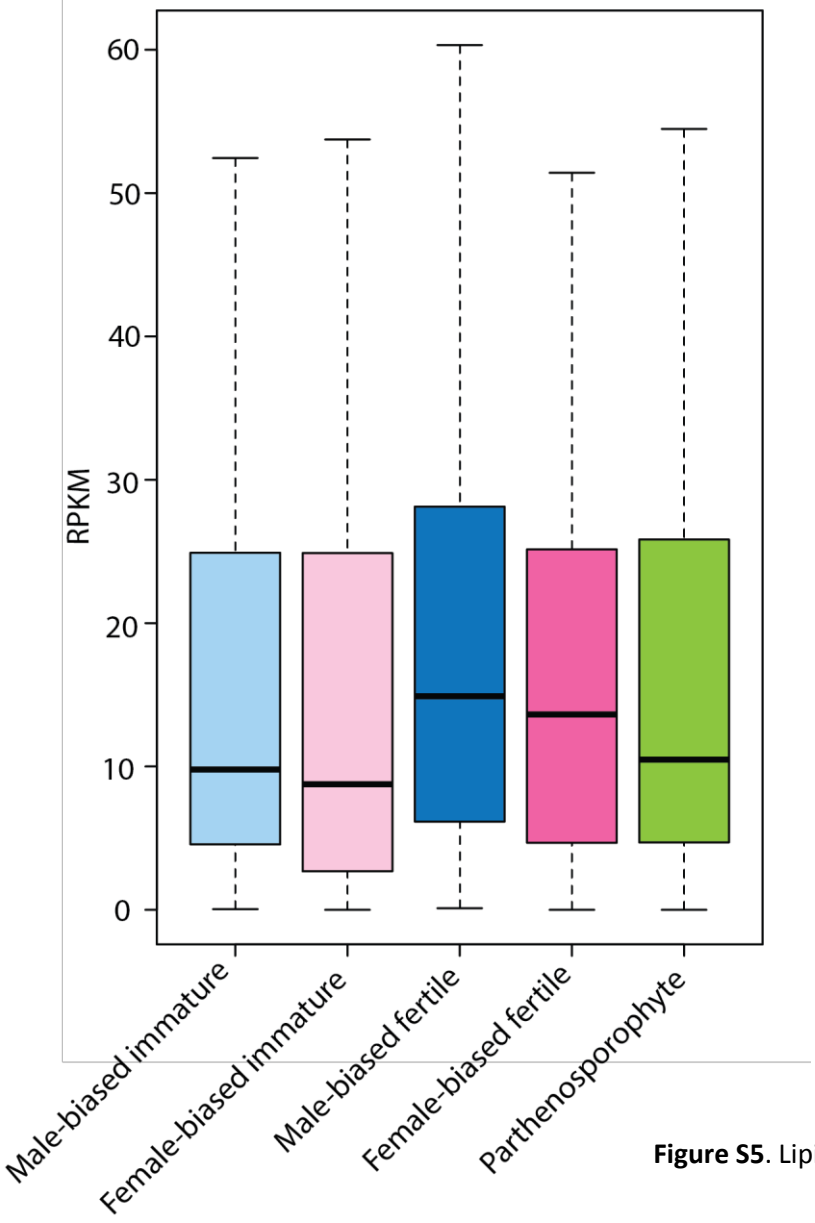

Figure S5. Lipinska *et al.* 2014

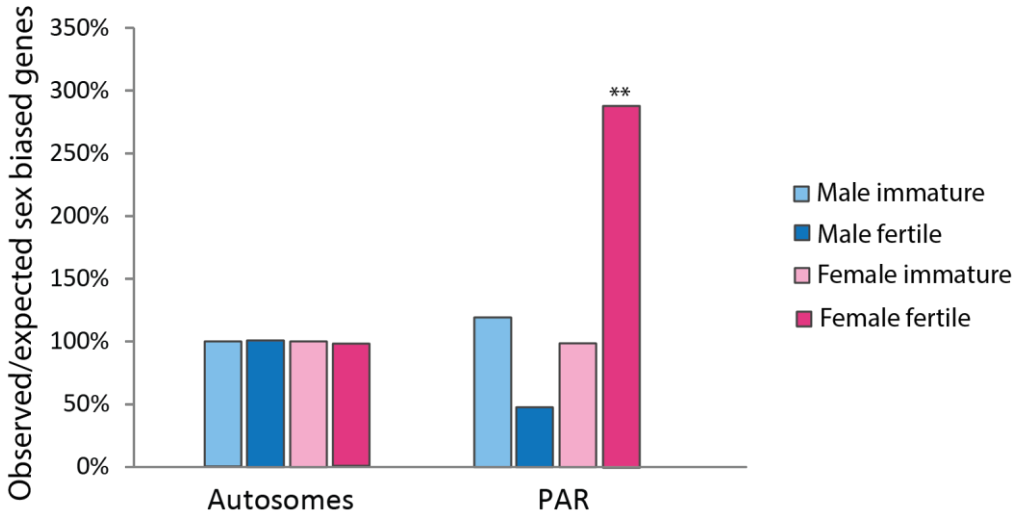

Figure S6. Lipinska *et al.* 2014

SUPPLEMENTAL FIGURES

Figure S1. Venn diagram showing the overlaps of sets of genes expressed (RPKM >1) in immature and fertile male and female gametophytes.

Figure S2. Venn diagram showing the overlaps of sets of sex-biased genes (RPKM >1, FC >=2, p_{adj} <0.1) expressed in immature and fertile male and female gametophytes.

Figure S3A. Mean gene expression levels (RPKM) at several degrees of sex-bias (from FC>1 to FC>10) for female (pink) and male-biased (blue) genes in fertile and immature gametophytes. SDR genes were excluded from this analysis. **S3B**. Boxplot showing the mean expression levels (RPKM) of female and male-biased genes for immature and fertile gametophytes.

Figure S4. Codon usage bias in sex-biased versus unbiased genes. Ribosomal genes were used as a reference for codon usage. Numbers represent mean values for each category. (A) Codon Adaptation Indexes (CAI); (B) Effective Number of Codons (ENC); $P_{()}$ - value of two-tailed Mann-Whitney test for comparisons between male- (M), female- (F), and unbiased (N) genes.

Figure S5. Enrichment of sex biased genes across the *Ectocarpus* genome. Ratio of observed to expected frequency of sex-biased genes on *Ectocarpus* autosomes and pseudo-autosomal region of the sex chromosome (LG30) is shown. Female-biased and male-biased genes are marked pink and blue, respectively. Dark and light colours correspond to the mature and immature gametophyte stages, respectively. Chi squared tests were used to assess statistical significance (*p<0.05; **p<0.01; ***p<0.001).

Figure S6. Characteristics of the duplicated genes with only one duplicate being sex biased. Gene expression levels (RPKM) of the nonbiased counterparts are shown across different life stages.

Table S1: see attached Excel file "Supplemental Lipinska *et al*. 2014"; sheet "Table S1"

Table S2: see attached Excel file "Supplemental Lipinska *et al*. 2014"; sheet "Table S2"

Table S4: see attached Excel file "Supplemental Lipinska *et al*. 2014"; sheet "Table S4"

Species*	Strain reference	Isolation location
Ectocarpus siliculosus 1a	Rb1 x EA1 progeny	Naples, Italy
Ectocarpus sp. Peru 1c	Ec602, Ec603	Peru
Ectocarpus sp. Greenland 1c	CCAP 1310/214	Kapisigdlit, Godhåbsfjorden, West Greenland
Ectocarpus fasciculatus	CCAP 1310/13	Roscoff, France
Scytosiphon lomentaria	Slom	Asari, Japan

Table S3. Lipinska *et al.* 2014

Species	Strain	Stage	Sex	Library reference	Raw data	Clean data	Unmapped reads	% of mapped reads
Ectocarpus sp.	Ec32	Mature GA	Male	GPO-1	25 119 067	22 428 865	3 148 572	85,96
				GPO-2	26 873 490	23 642 187	3 209 589	86,42
			Female	GPO-3	21 005 896	18 668 732	2 082 406	88,85
				GPO-4	32 150 185	28 667 939	2 902 492	89,88
		Immature GA	Male	GBP-24	75 827 247	73 723 385	5 235 532	92,9
				GBP-25	93 562 945	90 903 680	5 617 973	93,82
			Female	GBP-22	80 602 259	78 459 187	4 711 520	94
				GBP-23	85 541 801	83 125 361	5 188 704	93,76
		pSP		GBP-7	37 221 214	37 018 065	1 932 661	94,26
				GBP-8	29 670 293	29 491 668	1 659 306	93,81
		Upright filaments		GBP-18	32 080 985	31 431 264	1 374 924	97,97
				GBP-19	34 753 366	34 100 415	1 395 775	98,12
		Basal filaments		GBP-16	35 017 809	34 355 456	1 605 718	98,11
				GBP-17	32 818 363	32 207 868	1 519 686	98,14
Scytosiphon lomentaria	*Slom*	GA	Male	GPO-17	106 655 704	96 442 424	83 253 911	86,32
				GPO-18	69 382 687	63 429 391	55 184 477	87
		GA	Female	GPO-15	75 229 308	69 130 102	60 420 559	87,4
				GPO-16	93 124 633	84 881 043	73 668 696	86,79

Table S5. Lipinska *et al.* 2014

SUPPLEMENTAL TABLES

Table S1. Sex-biased genes in fertile and immature gametophytes indicated by DESeq program (FC >=2, p_{adj} <0.1).

Table S2. Gene Ontology terms significantly enriched among sex-biased genes (Fisher exact test, FDR <5%).

Table S3. Ectocarpales species used in positive selection analysis (PAML). Lineages of *Ectocarpus* are based on Stache-Crain *et al.* 1997.

Table S4. Positive selection analysis (PAML4, codeml) of sex-biased genes based on sequence alignments of *E. siliculosus* lineage 1a*, E.* siliculosus lineage 1c Greenland*, E.* siliculosus lineage 1c Peru*, E. fasciculatus, S. lomentaria*.

Table S5. Sequencing data statistics.

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III. Discussion and perspectives

Since the first description of *Ectocarpus* in 1819 by Lyngbye, very few sexual dimorphisms were identified for *Ectocarpus*. As described in the general introduction, differences between males and females were described for gametes at the levels of physiology, behaviour (Berthold, 1881; Müller, 1972) and capacity to develop through parthenogenesis (Berthold, 1881). In this study we were able to identify several additional sexual dimorphisms: gamete size, gametophyte habit and gametophyte fertility. We also analysed male and female transcriptomes (RNA-seq) allowing a study of sex-biased genes (SBGs) in a UV system, their evolutionary fate, and their genomic distribution. In *Ectocarpus* the proportion of SBGs is modest and SBGs also show a strong ontogenetic effect, with a higher number of SBGs at the immature stage compare to the mature stage.

The number of SBGs was higher in immature gametophytes than in mature gametophytes. In immature gametophytes the number of SBGs in male reaches 8.22% and 4.62% in females. This strongly contrasts with the proportions of SBGs (2.25% for male and 1.23% for female) in mature gametophytes, when sexes produce gametes and exhibit fully their morphological sexual dimorphisms. There is thus a strong ontogenetic effect on the number of SBGs. The ontogenetic effect observed is probably due to the early establishment of molecular actors involved in sexual differentiation before the full phenotypic expression of sexual dimorphisms at maturity. The establishment of sex differences involves a complex cascade of molecular reactions and it is possible that the establishment of those sexual differences involves more molecular actors than their maintenance. An effect of ontogeny on SBG expression patterns and evolutionary changes has been previously suggested for birds (Mank, 2009) and described in *Drosophila* (Perry *et al*., 2014).

In the study of Perry *et al*. (2014) it was also shown that *Drosophila* male-biased genes have a stronger tendency to retain more their sex-biased expression throughout development compared to female-biased genes. Even if expression of the large majority of SBGs in *Ectocarpus* is not maintained between immature and mature stages, similar pattern than in *Drosophila* is observed with more male-biased genes retaining their sex-biased expression in *Ectocarpus*. Indeed, 12% of the male-biased genes in immature are conserved in mature and only 3% for female-biased genes. This differential of conservation of expression between male and female-biased genes was already proposed to be probably the sign of an

earlier onset of male gametogenesis (Perry *et al*., 2014), which is also concordant with our observation that in *Ectocarpus* males produce their gametes earlier than females (Figure 6).

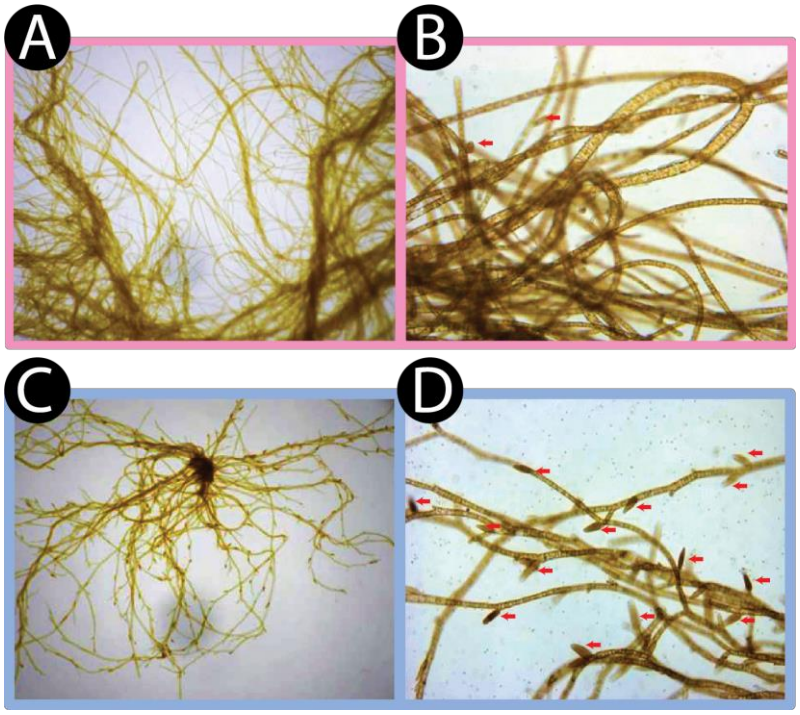


Figure 6. Representative photographs of mature gametophytes. Ec602 female gametophytes at maturity (**A** and **B**) and Ec603 male gametophytes at maturity (**C** and **D**). Red arrows indicate plurilocular gametangia (organs bearing gametes).

Another interesting pattern observed in *Ectocarpus* is the enrichment of female-biased genes on the PARs compare to male-biased genes. This feminization of *Ectocarpus* sex chromosomes is difficult to explain, but the model developed in Chapter 3 could explain such feminization if female-biased genes are also generation-biased genes.

Our idea of how sexual dimorphisms are shaped by sexual selection, mainly stems from studies in animals, where sexual dimorphisms are often ostentatious. A comparative transcriptomic analysis of several brown algae with different level of sexual dimorphism would allow to test the link between level of sexual dimorphism and proportion of genes having a SBG expression and provide us an idea of how much brown algae can differentially regulate gene expression between sexes. This would provide information about the scale of SBG in brown algae and could be further used as a reference to determine if the 12% of SBG in *Ectocarpus* is a relatively high proportion compared with other brown algae. For instance, we would expect a higher proportion of SBG in a strongly dimorphic brown alga such as *Laminaria*, where females produce few but large gametes and males produce tiny and

numerous gametes that are released into the surrounding medium. This difference in gamete production behaviours probably provides a scope for male-male competition for the access to female gametes. Therefore, differential investment in gamete production between female and male gametophyte of *Laminaria* probably increases the strength of sexual selection compared to *Ectocarpus*, possibly increasing the level of SBG. The brown algae represent a fascinating group for studies of the evolution of sexual dimorphism, specifically gamete size dimorphism, as this group exhibits a high level of variability for this trait, ranging from isogamous, through anisogamous, to oogamous systems (Luthringer *et al*., 2014). It would be interesting to generate and analyse genome sequences for several brown algae with different levels of gamete sexual dimorphism to investigate how sex chromosomes co-evolve with major changes in sexual dimorphism.

A particularly interesting trait that has been shown to differ between male and female gametes is parthenogenetic capacity. Indeed, in some *Ectocarpus* lineages, it has been noted that female gametes are able to develop into partheno-sporophytes, but male gametes cannot, and therefore that parthenogenesis is a dimorphic trait is these lineages (Berthold, 1881). This is a very striking feature because it suggests that there is a potential link between the sex determining region and the capacity to go through parthenogenetic reproduction. The next chapter focuses on the genetic basis of the association between sex and parthenogenesis capacity.

Chapter 5. Genetic Basis of Parthenogenesis, a Sexual Dimorphic Trait in *Ectocarpus siliculosus*

I. Introduction

The vast majority of eukaryotes reproduce sexually and only approximately 1‰ of eukaryotic multicellular taxa are asexual (Simon and Delmotte, 2003). This dominance of sexuality over asexuality is one of the major questions of evolutionary biology and is known as the "paradox of sex" (Maynard Smith, 1978; Williams, 1975). Indeed, in asexual population each individual is producing offspring which contrasts with sexual population where only half of the individuals (females) are producing offspring (see Chapter 1 section I.c). Therefore within a sexual population, an asexual individual would easily spread the capacity to reproduce asexually (Maynard Smith, 1978). However, in eukaryotes, it is sexual reproduction that dominates despite its two-fold cost (see Chapter 1 section II.c). Asexuality is a mode of reproduction by which offspring originate from a unique parent, inheriting exactly the same genetic information as the parent. This mode of reproduction is expected to be an evolutionary dead-end, because of the low capacity to produce evolutionary novelties. The phylogenetic distribution of asexual lineages tends to confirm this idea. Indeed, most asexual lineages occupy the terminal nodes of phylogenetic trees (Simon and Delmotte, 2003). This phylogenetic distribution of asexuality also suggests that asexuality evolved independently and repeatedly. The famous case of bdelloid rotifers is an exception to this evolutionary dead-end. The entire class of bdelloid rotifers is asexual, suggesting a stable reproduction strategy, which has led to them being referred to as an "evolutionary scandal" (Maynard Smith, 1986).

Asexuality has several possible modalities including for example fission, budding, vegetative reproduction and parthenogenesis. We will focus on the latter, which is defined as the development of a gamete without fertilization. In the animal and plant kingdoms, this process generally involves the gametes that have the largest energy reserve, namely female gametes, as this reserve is required to ensure parthenogenetic development. However in near-isogamous brown algae species usually both, male and female gametes, are capable of parthenogenesis. In anisogamous brown algal species only the female gametes are parthenogenetic (*i.e.* in the latter parthenogenesis is a sexually dimorphic trait). Exceptions to

this trend do however exist, *e.g*. *Desmarestia* where occasionally spermatozoids are capable of parthenogenesis (Ramirez *et al*., 1986). In many oogamous brown algae species neither the male nor the female gametes undergo parthenogenesis (especially in the Fucales), but in Laminariales there are some notable exceptions. Indeed *Laminaria angustata* is an oogamous species where unfertilized gametes can reproduce parthenogenetically (Motomura, 1991). Interestingly, *L. angustata* eggs have flagella remnants suggesting that the gametes of this species may be considered to represent an intermediate state between anisogamy and oogamy (Motomura and Sakai, 1988). One interesting possibility that would merit further investigation is that the flagella remnants may play a role in female parthenogenesis in these species, by allowing the formation of centrosomes in the unfertilised gamete. Overall, these trends suggest that gamete size influences parthenogenetic capacity up to a point, but that in oogamous species the large female gamete is specialised for zygote production and is no longer capable of initiating parthenogenetic development (Luthringer *et al*., 2014).

In the previous chapter we identified several sexual dimorphisms in *Ectocarpus*, including gamete size. Gametes are also involved in another sexual dimorphism, the capacity to reproduce through parthenogenesis. Indeed, in some *Ectocarpus* species such as the Peruvian species (1c lineage Stache-Crain *et al*., 1997), both female and male gametes have the capacity to undergo parthenogenesis (Bothwell *et al*., 2010), while in another species, *E. siliculosus* (1a lineage), only female gametes are able to develop through parthenogenesis (Berthold, 1881). In this study, we took advantage of the fact that parthenogenetic capacity is a sexually dimorphic trait in *E. siliculosus*, to analyse the genetic basis of parthenogenesis. A mapping-by-sequencing approach, termed SHOREmap (Box 4; Schneeberger *et al*., 2009), was employed with the aim of mapping the parthenogenesis locus. Finally, we performed a survey of parthenogenesis capacity in several species and populations of *Ectocarpus* from around the world to understand how common this sexual dimorphism was across populations.

II. Material and Methods

Brown algal culture

Ectocarpus strains were cultured in autoclaved natural sea water supplemented with half strength Provasoli solution (Starr and Zeikus, 1993) at 13°C, with a light:dark cycle of 12h:12 (20 µmol photons m^{-2} s^{-1}) using daylight-type fluorescent tubes. All manipulations

were performed under a laminar flow hood in sterile conditions. Annexe 2 presents all the algal strains used.

Measurement of parthenogenetic capacity

Gametophytes of several strains were grown as described above. When gametophytes were mature, release of gametes from the plurilocular gametangia was induced as described above. When gametes were released, the gametophyte was removed to a new Petri dish. Gamete germination was observed every 2 days. Gametophytes were considered as parthenogenetic (P+) when their gametes were able to develop into partheno-sporophytes with more than 10 cells. In contrast, gametophytes releasing gametes producing less than 10 cell partheno-sporophytes were phenotyped as non-parthenogenetic (P-).

Preparation segregating populations

A cross between a parthenogenetic female strain (Ea1; P+) and a non-parthenogenetic male strain (Rb1; P-) was carried out and a diploid heterozygous zygote was isolated (Ec236) (Figure 7). At maturity, Ec236 produced unilocular sporangia where meiosis took place. A total of 1900 unilocular sporangia were isolated. From each unilocular sporangium one gametophyte was isolated, which allowed the generation of a segregating population of 1900 gametophytes. Those 1900 gametophytes, named Ec236-1 to Ec236-1900, were cultivated as described previously but with supplementation of the seawater medium with an antibiotic solution (5ml/l), as described in Coelho *et al*., (2012a), to ensure absence of bacteria. Strains used are described in annexe 2.

DNA extraction and phenotyping the segregating population

The parthenogenetic capacity of the gametes was analysed from a subset of 274 gametophytes of the segregating population, as described above. The remaining 1600 gametophytes were maintained in stock for the fine mapping analysis. After phenotyping, each of the 274 gametophytes was frozen in liquid nitrogen in 96 well plates. After lyophilization, tissues were disrupted by grinding. DNA of each gametophyte was extracted using the NucleoSpin® 96 Plant II kit (Macherey-Nagel) according to the manufacturers' instructions and stored at -80°C. Sexing of gametophytes was carried out using two molecular sex markers for each sex (Annexe 3). PCR was performed with the following reaction

temperatures: 94°C 2min; 30 cycles of 94°C 40s, 60°C 40s and 72°C 40s; 72°C 5min, and with the following PCR mixture 2 µL DNA, 100 nM of each primers, 200 µM of dNTP mix, 1X of Go Taq® green buffer, 2 mM of $MgCl_2$, 0.2 µL of milk at 10% and 0.5 U of Taq polymerase (Promega).

In order to assess phenotype stability, new clonal gametophytes were produced from partheno-sporophytes of each strain, which were induced to produce unilocular sporangia.

Sequencing genomic data

DNA from individuals bulked according to their phenotype was needed for the SHOREmap analysis. P+ and P- bulks were prepared by pooling the DNA of 175 P+ individuals and the DNA of 78 P- individuals. In order to pool approximately the same amount of DNA for each individual, DNA was quantified using a NanoDrop 2000 spectrophotometer (ThermoScientific). Each bulk of DNA was purified using the NucleoSpin® from step four of the manufacturers' instructions and quantified using the Picogreen assay (Invitrogen). P+ and P- bulks of DNA were sequenced by Fasteris (CH-1228 Plan-les-Ouates, Switzerland) using the Illumina technology. Hundred-base-pair paired-end reads were sequenced and generated 88104906 and 105536895 reads for P+ and P- bulk respectively.

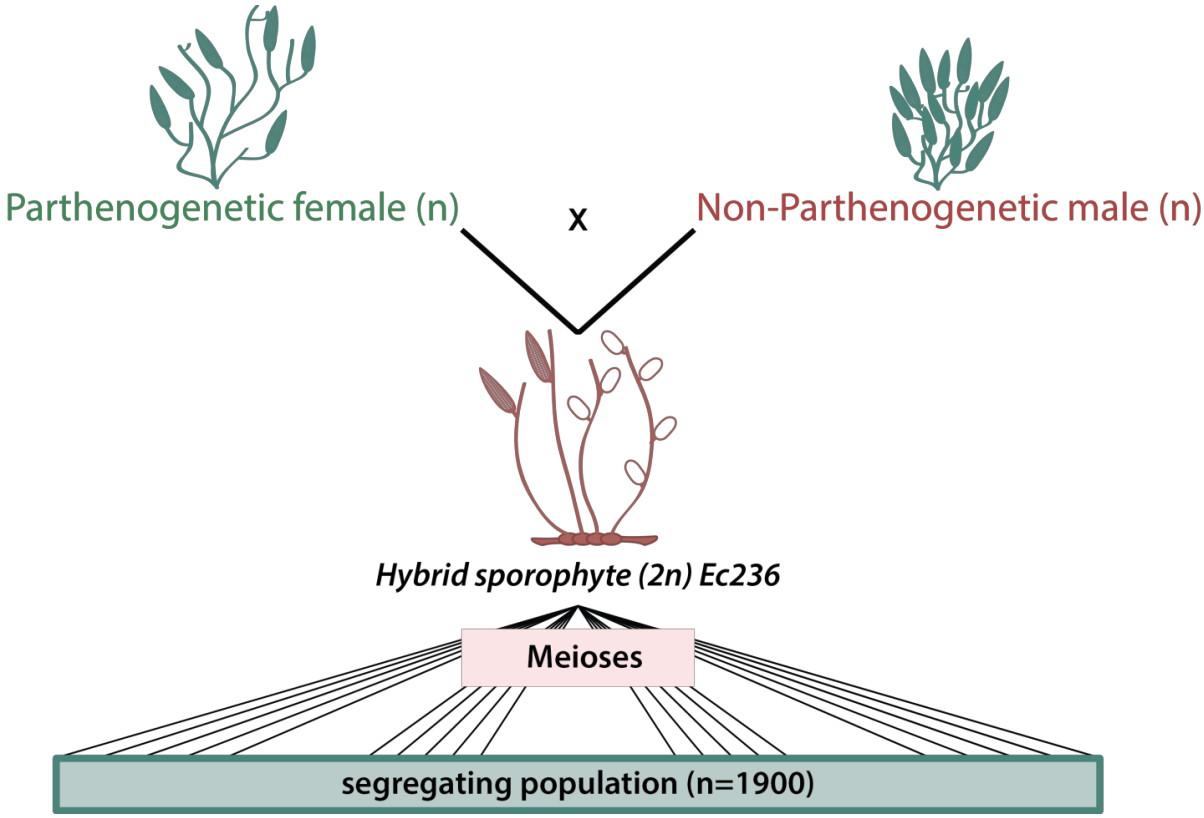


Figure 7. Parthenogenesis mapping population. Crossing a parthenogenetic female (Ea1) with a non-parthenogenetic male (Rb1) to produce a hybrid diploid sporophyte (Ec236) from which 1900 gametophytes (from independent meiosis events) were isolated. From approximately 300 gametophytes, parthenogenetic capacity was assessed, sexed and used for the SHOREmap analysis (Box 4).

SHOREmap analysis

Prinseq software was used to remove reads with an average quality of < 25, reads shorter than 50 bp, to trim sequences from 3' end by quality <20 and to remove sequences with other characters than A,T,G,C or N from each of the sequenced libraries (P+ and P-). The filtered and trimmed libraries were mapped on the hybrid male and female reference genome using Bowtie2 software with the "--very-sensitive" option and adjusting the number of seed extensions that can "fail" in a row before the software terminates (option -D) to 40. From the mapping we used the SHORE consensus to identify polymorphisms in each library compared to the reference genome. We then filtered the polymorphisms to remove polymorphisms specific to the *E. siliculosus* strains and to keep polymorphisms with frequencies of between 0.8 and 1.2. Finally those polymorphisms were used to run

SHOREmap outcrosses for allele frequency analysis across the full genome and only polymorphisms with scores higher than 25 were considered.

Genotyping candidate regions

Primers for CAPS markers were designed using the Sol Genomics Network (SGN) web-tool (Bombarely *et al*., 2011) and Primer3 (version 4.0.0). Specificity of amplification was tested using genomic DNA of males and females from the segregating population. Eight CAPS markers were designed on supercontigs (sctg) surrounding the *Ectocarpus* SDR and tested on the complete, phenotyped segregating population: one marker on sctg_357; 427; 105, two CAPS markers on sctg_285 and three on the sctg_242. Each PCR product was purified using the Montage PCR_{96} Cleanup kit (Millipore) in order to avoid genotyping errors that can be generated by PCR reagents during enzymatic digestion. Touch-down PCR was performed with the following reaction temperatures: 95°C for 5min; 13 cycles of denaturation at 95°C for 35s, annealing at 65–52°C for 35s and extension at 72°C for 1min 15s; then 27 cycles of denaturation at 95°C for 35s, annealing at 52°C for 35s and extension at 72°C for 1min 15s; with a final extension step at 72°C for 10min and with the following PCR mixture 1µL DNA, 100 nM of each primers, 200 µM of dNTP mix, 1X of Go Taq® colorless buffer, 2 mM of $MgCl_2$, 0.2 µL of milk at 10% and 0.5 U of Taq polymerase (Promega). Enzymatic digestion was performed with the following reaction temperature: 37°C for 12 hours and then 65°C for 20 min. The enzymatic reaction mixture was as follow: 0.1 µL of restriction enzyme, 1 µl of enzyme-dependent buffer and 10 µL of purified PCR product. Finally for sctg_251, linkage with the parthenogenesis locus was tested by sequencing a 580 bp fragment of the sctg. The PCR reaction was performed as described above and the sequencing reaction performed using the primers that has been used in the PCR reaction

Fitness measurement

Reproductive success was measured in the segregating population used for the SHOREmap analysis by measuring the capacity of male P+ and P- gametes to fuse with female gametes and by measuring the length of zygotes produced by crossing P+ or P- males with females. For this, males and females were crossed as described in Coelho *et al*. (2012) and the proportion of gametes that succeed in fusing was measured (‘functional gametes’, as described in Lovlie and Bryhni, 1976). The length of zygotes was monitored over the subsequent days using image analysis (ImageJ 1.46r Schneider *et al*., 2012). For the

measurement of the proportion of functional gametes, between 50 and 150 individual were counted for each cross, and five different P+ males (Ec236-34 and -245) and P- males (Ec236-10 and – 298) were crossed with several females (Ec236-39; -203; -233; -284 and Ec560). The lengths of zygotes produced after a cross between the female Ec560 and a male P- (Ec236-10) or a male P+ (Ec236-34) were measured after 5 hours, 24h, 48h, 3 days and 5 days of development. Statistical tests were performed using GraphPad Prism software. The difference between the percentages of functional gametes of male P- and P+ was statistically tested using a t-test (α=5%), after having checked that data met the assumptions of normality and homoscedasticity. The latter assumptions were not met for zygote length, and consequently the statistical significance of differences at each time of development was tested using a Mann Whitney U-test (α=5%).

Measurement of gamete size

Male and female gamete size was measured in the segregating population used for the SHOREmap analysis. Synchronous release of gametes from 3-4 week old cultures was induced by transferring ten gametophytes to a humid chamber in the dark for approximately 14 hours at 13°C followed by the addition of fresh PES-supplemented NSW medium under strong light irradiation. Gametes were concentrated by phototaxis using unidirectional light, and collected in Eppendorf tubes. Gamete size was measured by impedance-based flow cytometry (Cell Lab QuantaTM SC MPL, Beckman Coulter®). Gamete size was measured for a representative of each parthenogenetic phenotype found in the segregating population (P+ and P-): the P+ female Ec236-203 (n=1066), P+ male Ec236-210 (n=9755) and P- male Ec236-10 (n=45294). The values of gamete size shown represent the mean ±s.e. for each individual. A one-way ANOVA followed by several t-tests (α=5%) for pairwise comparisons was performed using GraphPad Prism software to compare female and male gamete size.

III. Results

Phenotypic characterization

The capacity of unfertilized gametes to develop parthenogenetically to form a partheno-sporophyte (pSP) was followed for a month for two strains of *E. siliculosus*, the female Ea1 and the male Rb1. After four days of parthenogenetic development male and female gametes exhibited approximately the same growth rate with the majority of gametes at

the 3-4 cell stage. After a month more than 94% of the female gametes had developed into functional partheno-sporophytes, with prostrate cells and upright filaments (>10 cells). In contrast, 96% of male gametes arrested their parthenogenetic development before reaching the 10 cells stage. Therefore the female is parthenogenetic (P+) and the male, which arrests parthenogenetic development after less than five cell divisions, is non-parthenogenetic (P-) (Figure 8).

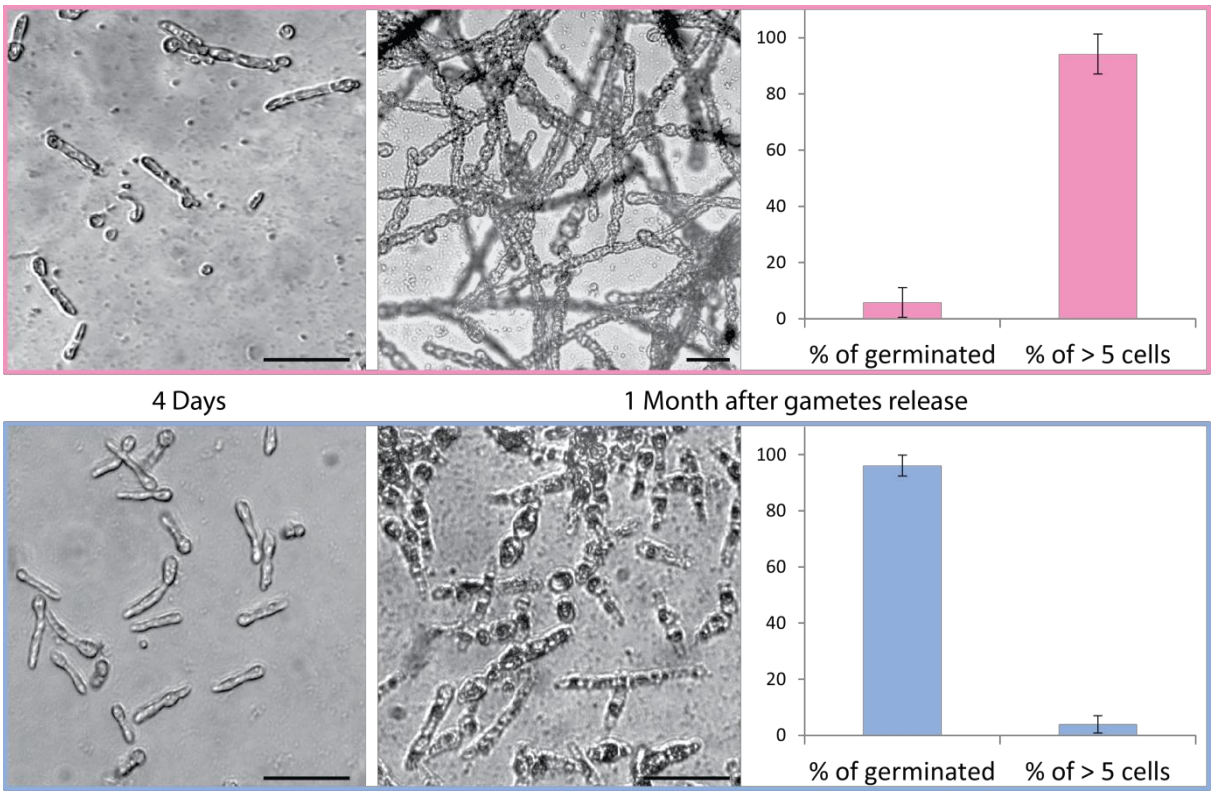


Figure 8. Parthenogenetic and non-parthenogenetic phenotypes. Left panel: young partheno-sporophyte after 4 days of development of parthenogenetic female (top) and non-parthenogenetic male (bottom). Middle panel: After a month, partheno-sporophytes from females' gametes are fully developed (top) and partheno-sporophytes from males' gametes remain at approximately 5 cells stage (bottom). Right panel: proportion of germinated and >5 cells partheno-sporophytes after a month of development for female gametes (top) and male gametes (bottom).

Analysis of a segregating population

We used a genetic approach to identify the parthenogenesis locus in *E. siliculosus*. Previous work on field collected *E. siliculosus* suggested that parthenogenetic capacity was partially sex-linked, as field collected male strains were consistently incapable of parthenogenesis in contrast to female strains. We crossed a male P- strain with a female P+

strain and constructed the Ec236 segregating population (Figure 7). Gamete germination patterns were analysed for 272 gametophytes of the Ec236 segregating population using microscopy. Eighty-four individuals were identified as non-parthenogenetic (P-) and 188 as parthenogenetic (P+). Using molecular sex markers, 128 males and 144 females were identified showing that this segregating population had a normal sex ratio that was consistent with a 1:1 segregation (chi2 test: p-value=0.36) (Table 1).

		Female	Male	Total
Ec 236 Population	P+	144	44	188
	P-	0	84	84
	Total	144	128	272

Table1. Contingency table. Phenotyping and sexing summary of the parthenogenesis segregating population Ec236 population. P+: positive parthenogenetic capacity; P-: negative parthenogenetic capacity. In grey individuals used for the calculation of genetic distance between SDR and parthenogenesis capacity.

Recombinants between the SDR and the parthenogenesis locus were consistently P+ males and no female recombinants (female P-) were found. The absence of females can be either due to the lethality of P- allele for females or to an epistasis effect between parthenogenesis locus and female SDR where regardless of the parthenogenesis allele associated with the female SDR, females are capable of parthenogenesis. In both cases the absence of P- females induces a distortion of segregation, therefore only males were used to calculate the genetic distances. With 44 recombinants (P+ males), in a total population of 128 males, the genetic distance between SDR and parthenogenesis locus could be estimated at 34 cM (number of recombinants x 100 / total population) indicating that there is partial genetic linkage between the two loci, and therefore suggesting that the parthenogenesis locus is located in the recombining region (PAR) of the sex chromosome.

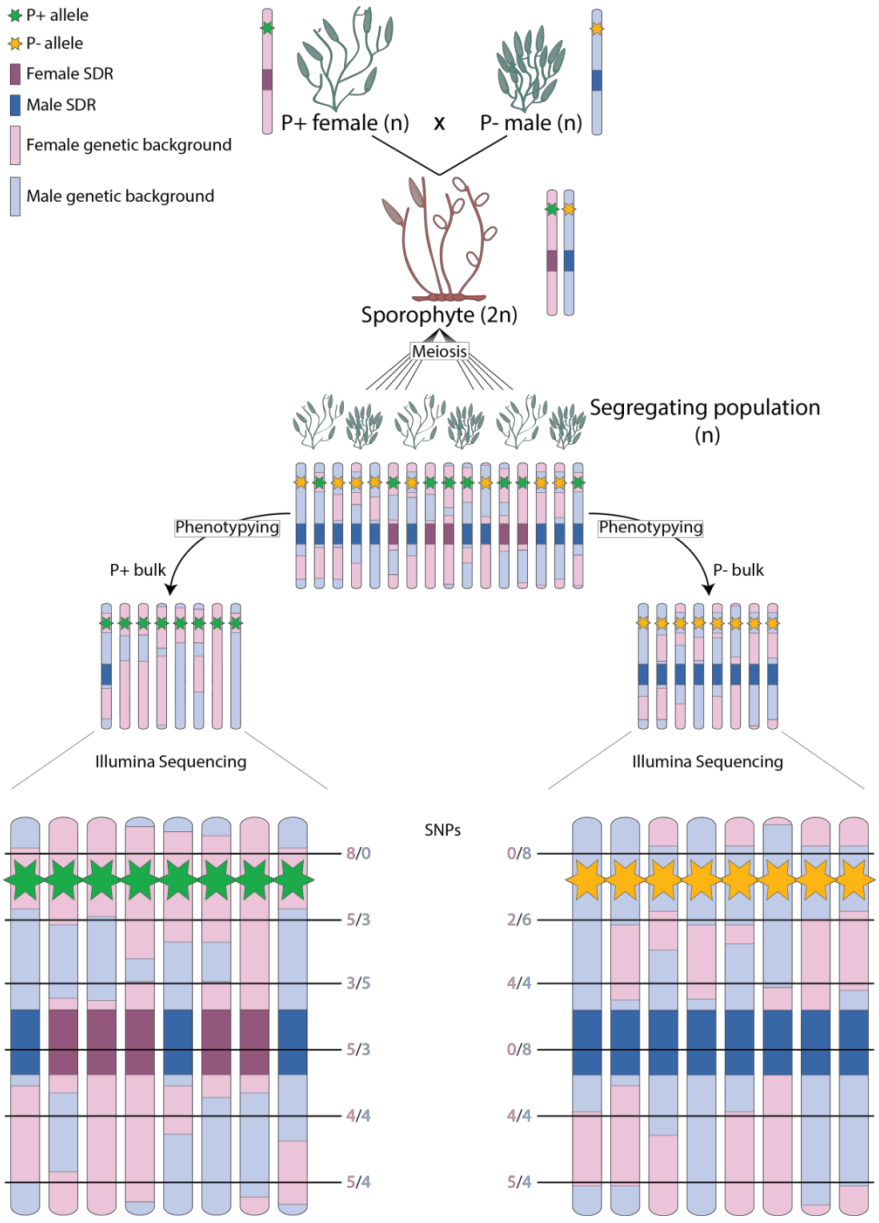
Parthenogenesis is a genetically controlled trait

To further confirm the genetic character of parthenogenesis, the Ec236 population crosses between a P+ male (Ec236-202) and a P+ female (Ec236-91) were performed to

produce two diploid sporophytes (Ec620 and Ec696) from which 23 gametophytes (8 males and 15 females) were produced and phenotyped for parthenogenetic capacity. All gametophytes produced gametes that were capable of parthenogenesis, which confirms the genetic character of the parthenogenesis phenotype.

Box 4: SHOREmap principle

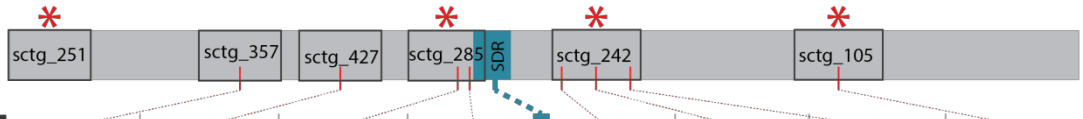
Schematic representation of the SHOREmap approach. One parthenogenetic (allele P+) and a non-parthenogenetic (allele P-) haploid parents were crossed to form a diploid heterozygous sporophyte from which, after meiosis, a segregating population was isolated. After having analysed the parthenogenetic capacity of each individual from the segregating population, DNA of each individual was pooled in order to construct a P+ bulk and a P- bulk. Each bulk was sequenced in order to identify markers and analyse their segregating pattern (*in silico*) at a genome-wide scale.



SHOREmap analysis indicated candidate regions preferentially on the PAR

DNA samples from 175 P+ strains (137 females and 38 males) and 78 P- strains (all male) were pooled to form the P+ and P- bulks, respectively. More than 60% of the reads from each of these two bulks mapped onto the Peruvian *Ectocarpus* reference genome, suggesting that Neapolitain strains and the Peruvian strain were close enough to use the latter as a reference. Polymorphisms (SNPs, insertions and deletions) were identified using the SHORE software (Ossowski *et al*., 2008) (see Box 4). Only markers common to the two bulks with a frequency of between 0.8 and 1.2 and markers unique to each bulk with a frequency of between 0.1 and 0.9 were retained for the SHOREmap analysis. Visualization of allele frequencies across the genome using SHOREmap allowed the identification of several candidate regions on the recombining region of the sex chromosome, within supercontigs (sctg)_285 (between positions 148000 and 153999), sctg_251 (39000 and 48999), sctg_105 (216000 and 224999), sctg_242 (110000 and 119999) and also some autosomal supercontigs such as sctg_8 (550000 and 639999), sctg_211 (197000 and 205999), sctg_324 (91000 and 95999). Based on those results and on the availability of *E. siliculosus* P+ and P- sequences, eight CAPS markers were designed for the candidate regions of the PAR (except the sctg_251) and for two additional PAR supercontigs, sctg_357 and sctg_427. For the candidate region on sctg_251 enzymatic digestion did not allow to discriminate any genotype, therefore linkage to the parthenogenesis locus of the sctg_251 was tested by sequencing a fragment of sctg_251 from ten P+ males and ten P- males. The CAPS marker 357_caps (on sctg_357) was used to genotype 221 individuals from the 272 individuals of the segregating population. Forty-seven individuals presented a recombination event between the marker and the parthenogenesis capacity, which place the 357_caps marker at approximately 29 cM from the parthenogenesis locus. This weak genetic link between the marker 357_caps and the parthenogenesis capacity suggest that the latter is located on sex chromosomes. Genotyping the segregating population using the seven remaining markers tended also to indicate that the parthenogenesis locus was in the pseudoautosomal regions of the sex chromosome with all markers having a genetic distance lower than 40cM. The 105_caps marker was found to be the most distant from the parthenogenesis locus, with a genetic distance of 38 cM. The closest CAPS marker found in the analysis was the 285_caps_2 marker, which was 15 cM from the parthenogenesis locus (Table 2). Finally the sequencing of sctg_251 fragment did not allow any polymorphism linked to the parthenogenesis capacity to be identified, suggesting that the sctg_251 it is not linked to the parthenogenesis locus either. Therefore these CAPS markers

did not allow the candidate regions identified by the SHOREmap approach to be validated. However, the genetic distance between the parthenogenesis locus and the different markers was less than 40cM, indicating weak genetic linkage and suggesting that the locus responsible for the control of parthenogenesis is located on the sex chromosome. Interestingly, genotyping of the eight CAPS markers indicated that the relative genetic distances between the SDR and the different markers was conserved between the reference strain (see Chapter 3) and the *E. siliculosus* strains used in this study, which suggests that synteny is conserved between these two species.



Markers name	357_caps	427_caps	285_caps_2	285_caps_1	242_caps_1	242_caps_2	242_caps_3	105_caps
# recombinants (marker vs. P locus)	40	33	20	25	26	34	34	13
# non-rec. (marker vs P locus	61	62	44	39	56	67	65	36
% rec in cM (marker vs. P locus)	**39,6**	**34,7**	**31,2**	**39,1**	**31,7**	**33,7**	**34,3**	**26,5**
# recombinants (marker vs.SDR)	43	20	6	8	5	24	40	19
# non-rec. (marker vs.SDR)	178	193	151	131	162	200	179	49
% rec in cM (marker vs.SDR)	**19,4**	**9,4**	**3,8**	**5,7**	**2,9**	**10,7**	**18,3**	**27,9**

Table 2. Genotyping of CAPS markers. Genotyping of eight CAPS markers surrounding the SDR to estimate the genetic distance between each marker and parthenogenesis locus (P locus); each marker and SDR. On the top a schematic representation of the sex chromosome in *Ectocarpus* with the relative position of each sctg analysed. For the calculation of genetic distance between each marker and P locus only males were took into account (see Results section). Red asterisks indicate the candidate regions proposed by the SHOREmap approach. Red lines indicate the position of each marker on supercontigs.

Analysis of the fitness of P+ and P- male gametes

No P+ males have been found, to date, in natural *E. siliculosus* populations. To understand the causes of the absence in the field of a phenotypic class (P+ male) that is viable in culture under laboratory conditions, we compared the fitness of P+ and P- males. Crosses were performed between several females and either P- or P+ males, and we scored both the proportion of successful ma tings and zygote growth. Male P- gametes tended to fuse more

efficiently to with female gametes compared to the recombinant P+ male gametes (Figure 9, Student's t-test p=0,059). Also zygotic growth was significantly higher for zygotes derived from P- males than from P+ males and this difference was significant at any time between 5 hours and 5 days of development (Figure 9, all Mann-Whitney u-tests gave p<0,05). Taken together, these analyses demonstrated that recombinant P+ male gametes were less fit than P- male gametes suggesting that the P+ allele disfavours both males and their zygote progeny.

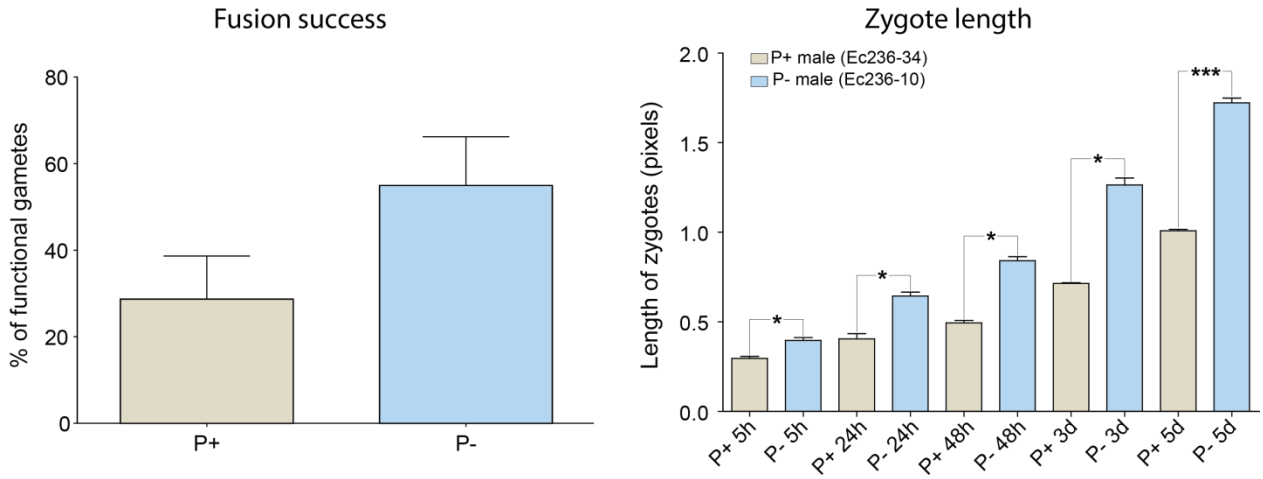


Figure 9. Fitness of parthenogenetic and non-parthenogenetic males. Males carrying the P- allele are fitter than P+ males. Fitness was measured by counting the proportion of zygotes after crosses with female gametes (left graphic; n=46). Fitness was also measured by following the growth of zygotes (from 5hours to 5 days after gamete release) from crosses performed between female and male P+ and male P-.

Gamete size and parthenogenetic capacity

In anisogamous and oogamous species gamete size is expected to influence parthenogenetic capacity. In order to assess the potential link between parthenogenetic capacity and gamete size in *Ectocarpus* we used various gametophytes from the mapping population (P- males, P+ males and P+ females) to measure gamete size (Figure 10). Two males with different parthenogenetic phenotypes (Ec236-210 P+ and Ec236-276 P-) produced gametes of almost the same size: 3.9µm of diameter. The third male (Ec236-10 P-) produced larger gametes (4µm of diameter) but these were still smaller than the female gametes (4.2µm of diameter; Ec236-203 P+). Therefore in the Ec236 population the capacity to do parthenogenesis does not depend on the size of the gametes.

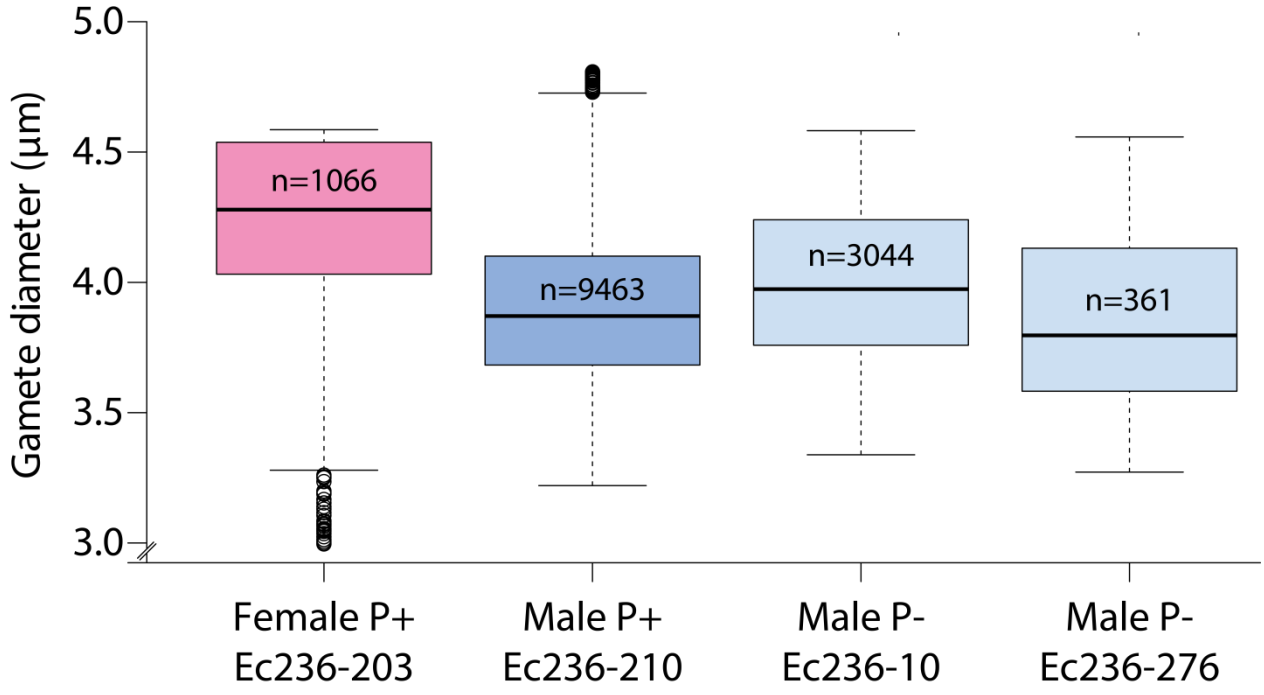


Figure 10. Gametes size of parthenogenetic female, male and non-parthenogenetic males. Mean diameter of female P+ (n=1066), male P+ (n=9436) and two males P- (n=3044 and 361) measured by flow cytometry. Error bars show standard errors.

Parthenogenetic capacity of diverse *Ectocarpus* strains and species

A survey was carried out to access the parthenogenetic capacity of gametes from several strains and species of *Ectocarpus* from around the world (Table 3). The *E. siliculosus* species was found in Naples (Italy) and in Perharidy (Roscoff, France). Capacity to do parthenogenesis in those strains were analysed from gametophytes produced from diploid sporophytes found in the field. The parthenogenesis capacity of those laboratory cultured gametophytes confirmed in nature the presence of both parthenogenetic phenotypes suggesting the presence of both parthenogenetic alleles (*i.e.* P+ and P-). The four strains of *Ectocarpus crouaniorum* (two of each sex) from field sporophyte of Perharidy (Roscoff, France) are capable of parthenogenesis. The capacity to do parthenogenesis was also found in both sexes in the reference sequenced strain from Peru, *Ectocarpus sp*. Finally two strains of *Ectocarpus sp*. (lineage 4) from Kaikoura (New-Zealand) show a sexual dimorphism for the capacity to do parthenogenesis with P+ female and P- male. Peruvian and New-Zealand strains were sampled more than twenty years ago and may however not be representative of actual populations (Table 3)

Species	Population	Strain name	Sex	Phenotype	# Phenotyped	Origin
E.siliculosus	Naples	021-1	Male	P-	1	From field SP
E.siliculosus	Naples	021-2	Female	P+	1	From field SP
E.siliculosus	Naples	022-1	Male	P-	3	From field SP
E.siliculosus	Naples	023-1	Female	P+	1	From field SP
E.siliculosus	Naples	023-2	Female	P+	1	From field SP
E.siliculosus	Naples	024-2	Female	P+	1	From field SP
E.siliculosus	Naples	025-1	Male	P+	1	From field SP
E.siliculosus	Naples	025-2	Female	P+	1	From field SP
E.siliculosus	Naples	127-1	Female	P+	2	From field SP
E.siliculosus	Naples	127-2	Male	P+	1	From field SP
E.siliculosus	Naples	128-2	Female	P+	1	From field SP
E.siliculosus	Naples	130-1	Male	P-	2	From field SP
E.siliculosus	Naples	130-2	Female	P+	2	From field SP
E.siliculosus	Naples	131-2	Male	P-	2	From field SP
E.siliculosus	Naples	133-2	Female	P+	1	From field SP
E.siliculosus	Naples	134-2	Male	P-	2	From field SP
E.siliculosus	Naples	135-1	Female	P+	1	From field SP
E.siliculosus	Naples	135-2	Female	P+	1	From field SP
E.siliculosus	Naples	136-1	Female	P+	2	From field SP
E.siliculosus	Naples	136-2	Female	P+	2	From field SP
E.siliculosus	Naples	137-2	Female	P+	1	From field SP
E.siliculosus	Naples	138-1	Female	P+	1	From field SP
E.siliculosus	Naples	139-1	Female	P+	1	From field SP
E.siliculosus	Naples	140-1	Female	P+	1	From field SP
E.siliculosus	Naples	140-2	Female	P+	1	From field SP
E.siliculosus	Naples	142-1	Female	P+	3	From field SP
E.siliculosus	Naples	143-1	Male	P-	1	From field SP
E.siliculosus	Naples	143-2	Female	P+	2	From field SP
E.siliculosus	Naples	147-2	Male	P+	1	From field SP
E.siliculosus	Naples	148-1	Male	P-	1	From field SP
E.siliculosus	Naples	149-2	Male	P+	1	From field SP
E.siliculosus	Perharidy (Roscoff)	110-1	Male	P-	2	From field SP
E.siliculosus	Perharidy (Roscoff)	110-2	Female	P+	2	From field SP
E.siliculosus	Perharidy (Roscoff)	111-1	Female	P+	1	From field SP
E.siliculosus	Perharidy (Roscoff)	111-2	Male	P-	2	From field SP
E.siliculosus	Perharidy (Roscoff)	118-1	Male	P-	2	From field SP
E.siliculosus	Perharidy (Roscoff)	118-2	Female	P+	1	From field SP
E.siliculosus	Perharidy (Roscoff)	120-2	Female	P+	1	From field SP
E.siliculosus	Perharidy (Roscoff)	122-1	Female	P-	2	From field SP
E.crouaniorum	Perharidy (Roscoff)	Ec ph11-s 2A-38-1	Male	P-	1	From field SP
E.crouaniorum	Perharidy (Roscoff)	Ec ph11-s 2A-38-3	Male	P-	1	From field SP
E.crouaniorum	Perharidy (Roscoff)	Ec Ph 11-s 2a-38-6	Female	P+	1	From field SP

E.crouaniorum	Perharidy (Roscoff)	Ec Ph11-s 2a-38-8	Female	P+	1	From field SP
Ectocarpus sp.	Peru	Ec32	Male	P+		Laboratory culture
Ectocarpus sp.	Peru	Ec87	Female	P+		Laboratory culture
Ectocarpus sp.	New Zealand	NZKU 1–3	Male	P-		Laboratory culture
Ectocarpus sp.	New Zealand	NZKU 32-22-21	Female	P+		Laboratory culture
E.siliculosus	Naples	Ea1	Female	P+		Laboratory culture
E.siliculosus	Naples	Rb1	Male	P-		Laboratory culture

Table 3. Parthenogenetic capacity in several populations/species of *Ectocarpus*. For each strain gametes germination was followed under microscope to determine their parthenogenetic capacity, either parthenogenetic (P+) or non-parthenogenetic (P-). When feasible parthenogenetic capacity was confirmed by several phenotyping. SP=Sporophyte.

IV. Discussion and perspectives

Epistasis with the SDR or female lethality of the P+ allele?

In the segregating population derived from the cross between the parthenogenetic and non-parthenogenetic *E. siliculosus* strains (Figure 7) all recombinants were males (males P+) and no female recombinants (female P-) were recovered. One plausible hypothesis is that combination of the female SDR with the P- allele is lethal, making the P- allele strongly deleterious for females. If this is the case, when gametophytes were selected to produce the segregating population, P- female meiospores would not have been able to develop into gametophytes and would not have been isolated. Potentially, under this hypothesis, parthenogenesis could be a sexually antagonistic gene. Since the sex-ratio in the segregating population is close to 50:50 (and even tends towards female-bias) this hypothesis is however unlikely to explain the absence of phenotypically non-parthenogenetic females. In fact, if a genetic association between female SDR and P+ locus was lethal for females (and not for males), we would have expected a male-biased sex ratio. Another possible explanation for the absence of P- females, could be that there is some form of epistasis, so that the parthenogenetic allele would not affect the parthenogenetic capacity if in association with the female SDR. In this case, all females would produce parthenogenetic gametes irrespective of the allele present at the parthenogenesis locus. If this hypothesis is correct, the SHOREmap approach may encounter some problems, which will be discussed in the next paragraph.

Rough mapping of the parthenogenesis locus

The SHOREmap analysis uses a genome of reference to map and assemble sequence reads and to identify polymorphisms across the genome. In theory, sequencing of one bulk of DNA (P+ or P- in this case) should be sufficient to map the genetic basis of a phenotype of interest using the SHOREmap approach. However, in this study P+ and P- bulks were needed for preliminary data processing. This is because the *E. siliculosus* strain used in this study to analyse the genetic basis of parthenogenesis is a different species from the reference strain that was sequenced to construct the reference genome. Therefore, sequence data for the two bulks of DNA was essential to eliminate from the analysis *E. siliculosus*-specific polymorphisms and to specifically analyse the segregation pattern of polymorphisms between P+ and P- bulks. CAPS markers designed to confirm candidate regions identified with the

SHOREmap approach did not allow any markers fully linked to the parthenogenesis locus to be found. However, the genotyping result using the eight CAPS markers designed on sex chromosomes strongly suggested that the parthenogenesis locus is located on the PAR. Some autosomal candidate regions identified by the SHOREmap approach still need to be tested. This technique has already been successfully used in our laboratory to identify a mutated locus in an *Ectocarpus* mutant line (unpublished data), but the mutation in question was in the same genetic background as the reference genome. The difficulties encountered in this study may be due to the fact that the species used for the reference genome is different from the SHOREmap species. A further complication may be the effects of the putative epistasis between the female SDR and the parthenogenesis locus. This is because, in the P+ pool of DNA, we might expect to find males and females that are genotypically P+ but also females that are genotypically P- but phenotypically P+. Therefore the P+ bulk of DNA that was sequenced would contain a mixture of P+ and P- genotypes, which would make the SHOREmap analysis unexploitable.

The different analysis performed to identify the parthenogenetic locus strongly indicated a role for the sex chromosomes. Therefore, more effort is required to design more markers along the entire length of the sex chromosome to search for genetic linkage to the parthenogenesis locus for each supercontig of the PAR. Additionnaly the three autosomal regions identified by the SHOREmap need to be tested to totally exclude the possibility that the parthenogenesis locus is autosomal.

A sexual-antagonistic parthenogenesis locus?

In populations where parthenogenesis is a sexually dimorphic trait (*E. siliculosus*), no P+ males have been found in field collected individuals. Moreover, fitness analysis showed that males carrying the P+ allele were less fit than males carrying the P- allele. The difference in fitness was quite striking, suggesting that there must be a reason why this allele is maintained in the population, and strongly suggesting that this is a sexual antagonistic locus. One possibility is that this allele is beneficial for females. This hypothesis is currently difficult to verify because P- females are either not viable and therefore unavailable (the hypothesis of P- being lethal for females), or phenotypically undistinguishable from P+ females (the epistasis hypothesis where the parthenogenesis locus does no influence the parthenogenetic capacity of females).

The other possibility is that this P+ allele may be advantageous also for males in some particular environmental situations. Indeed the capacity to reproduce parthenogenetically can be an advantageous strategy in the boundaries of distribution limit where the population density is low. In those cases, the chance of finding a gamete of the opposite sex is lower and asexual reproduction may be advantageous. The P+ allele would provide higher fitness for male gametes because they would be able to develop in absence of fusion with female gametes. Consistent with this hypothesis, it was shown that, at the limit of its range of distribution, the brown alga *Laminaria digitata* has a tendency to reproduce mainly parthenogenetically (Oppliger *et al*., 2014). It would be interesting to analyse several populations of *E. siliculosus* at different ranges of distributions to correlate the proportion of P+ males with male/female population density.

Molecular evolution tools can be used to test if the parthenogenesis locus shows the expected footprints of polymorphism due to sexually antagonistic selection: high diversity, and other evidence of balancing selection maintaining alleles polymorphic over a long evolutionary period (Qiu *et al*., 2013). Finally, if we have access to the SDRs of other brown algae showing varying levels of gamete dimorphism, it would be interesting to investigate if the parthenogenesis locus is located within the SDR of anisogamous brown algae where parthenogenesis is strictly correlated with sex (Luthringer *et al*., 2014). These investigations would not only improve our understanding of the mechanisms by which parthenogenesis operates, but would also provide much needed empirical evidence for the direct implication of sexual antagonistic genes in events that cause loss of recombination in the sex chromosomes.

The inheritance of mitochondria: a key feature for parthenogenesis capacity?

The molecular basis of parthenogenesis has been recently studied in *Scytosiphon lomentaria,* an isogamous species close related to *Ectocarpus,* using a proteomic approach (Han *et al*., 2014). In this species, as in *Ectocarpus*, it has been shown that, females always produce parthenogenetic gametes and some males produce non-parthenogenetic gametes that rapidly arrest their parthenogenetic development at the 4 cell stage. In this study Han *et al*. (2014) put forward an interesting hypothesis to explain the parthenogenetic trait in *S.lomentaria*. In this near-isogamous species after the zygotic four cell stage, male mitochondria start to be destroyed, and only female mitochondria are inherited by the next generation (Kimura *et al*., 2010). Han *et al*. (2014) hypothesized that the mechanism for the

control of uniparental inheritance of mitochondria may also control the parthenogenetic capacity of the male (the "mitochondrial" hypothesis). Indeed if a non-parthenogenetic male gamete does not find a partner with which to fuse, parthenogenetic development is initiated, but if the mechanism for the specific disappearance of male mitochondria is ongoing, the young partheno-sporophyte would lose its mitochondria, arresting parthenogenetic development. In *Ectocarpus* uniparental inheritance of mitochondria has also been reported (Peters *et al*., 2004a) but no study has been carried out on the timing of the male mitochondria loss. To test the "mitochondrial" hypothesis in *Ectocarpus*, the presence and integrity of mitochondria could be assessed by transmission electron microscopy in gametes and developing partheno-sporophytes of P+ and P- males.

Interestingly, we have noticed that non-parthenogenetic male gametes initiate parthenogenesis and then rapidly arrest development after about 5 cell divisions. This suggests that genetic and cellular components are present for the first parthenogenetic cell divisions but that subsequent cell divisions require other components to continue parthenogenetic development. We used therefore a pharmacological approach to investigate the cellular basis of parthenogenesis in *Ectocarpus*. These experiments are described in the next chapter.

Chapter 6. Insights into the cellular basis of parthenogenesis in *Ectocarpus*

I. Introduction

Brown algae have been widely used as models to study embryogenesis. This is because they offer a number of advantages such as the ease with which gametes and zygotes can be obtained and manipulated (contrary to land plants systems where the embryos are embedded in the sporophytic tissue). For instance, *Fucus* is particularly appropriate to study a large number of synchronous zygotes and to carry imaging, microinjection and biochemical analyses (Brownlee *et al*., 2001). The thorough characterization of polarization, germination and first cell divisions in this group of organisms clearly showed that early developmental processes are crucial in the determination of the correct patterning of the embryo and future adult plant (Brownlee and Bouget, 1998; Corellou *et al*., 2000). These studies, however, focused on organisms where a large female gamete (egg) is fertilized by a small male gamete (sperm) (oogamy). Not all brown algae are oogamous, and this group actually exhibits an exceptionally broad range of sexual systems, ranging from isogamy to oogamy with different degrees of sexual differentiation (Luthringer *et al*., 2014; Silberfeld *et al*., 2010). For example, in contrast to the situation in the Fucales, in many brown algae the male and female gametes have approximately the same size (near-isogamy). Interestingly, near-isogamy in brown algae is often associated with the capacity to develop parthenogenetically (Luthringer *et al*., 2014), *i.e*., a male or female gamete that does not meet a partner of the opposite sex, can still switch on a "zygotic" program on its own. The triggering of the sporophyte program is therefore independent of fertilization by a gamete of the opposite sex. In this case, the embryonic developmental program has to be initiated and sustained in the absence of the paternal or maternal genome.

The model brown alga *Ectocarpus* produces male and female near-isogametes and, at least in some *Ectocarpus* strains, both male and female gametes have the capacity to develop parthenogenetically. In *Ectocarpus* the sporophytic program is triggered when male and female gametes fuse but also when gametes do not find any partner to fuse with, and develop parthenogenetically. The developmental patterns of zygotic and parthenogenetic sporophytes

are strictly similar except that parthenogenetic development triggers the sporophytic program with a slight delay (Peters *et al*., 2008). Apart from this delay, the pattern of early development of a diploid sporophyte (zygote as the initial cell) and a partheno-sporophyte (gamete as the initial cell) appears to be largely similar (Peters *et al*., 2008). In both cases, germination is bipolar, and the two daughter cells exhibit a symmetric first cell division, producing the two ends of a prostrate filament. This symmetrical first cell division is followed by several other divisions which produce a prostrate filament after a few days. The cells of the prostrate filament become rounder and their cell walls thicken as they became older. Laterals with the same morphology as the initial filament are produced from the rounded cells, and grow along the surface of the substratum.

While an important amount of work has been published on the early stages of brown algal zygote development, less is known about the mechanisms regulating parthenogenetic development. The aim of our study was to characterize the early steps of parthenogenesis, in particular the dependence of the early stages of development of the *Ectocarpus* partheno-sporophytes on *de novo* transcription and translation. We hypothesised that the triggering of parthenogenesis would be dependent on *de novo* transcription and translation because of the small size of the gamete, which should preclude accumulation of large reserves of transcripts and proteins. Surprisingly, we found that the germination and first cell divisions of the partheno-sporophytes are uncoupled from *de novo* transcription, suggesting that early development relies exclusively in mRNA already present in the 4-µm gamete. Cells continued to develop in the absence of *de novo* transcription up to the 5-10 cell stage. Germination was also independent of *de novo* translation, suggesting that proteins necessary for germination are already present in the gamete. Translation was however necessary for the first cell division, indicating that new proteins must be translated during the first cell cycle. Our results, together with recently published work on the transcriptome of gametes of *Ectocarpus* (Lipinska *et al*., 2013), are consistent with the view that brown algal gametes contain the mRNA and proteins necessary for the very early steps of development. As gametes are one of the most fragile stages of the life cycle, they have probably evolved this strategy to increase their chances of survival in a harsh environment, where unfused gametes are cell-wall less and therefore exposed to biotic (predation) and abiotic factors.

II. Material and Methods

Algae material and culture

The strain Ec32 whose genome has been sequenced (Cock *et al*., 2010) was used in this study. Standard culture conditions were used as described in Coelho *et al*.. Briefly, gametophytes were grown in 140mm Petri dishes at 15°C, at a density of 10 individuals per Petri dish. Natural seawater (NSW) was filtered, autoclaved and enriched with half strength Provasoli solution (Starr and Zeikus, 1993). The maturity of gametophytes was accessed by microscopy. Synchronous release of gametes (time zero) was induced by transferring mature gametophytes from 13°C in the dark for five hours into strong light and adding fresh NSW enriched with Provasoli solution (PES in the following). Released gametes were transferred to a glass coverslip inside a Petri dish by pipetting. Development was followed every two days by counting at least one hundred individuals under an inverted microscope. Six categories were screened: round cells, germinated cells, two-cell, 3-5 cells, 6-10 and more than 10-cell stage. Three biological replicates and were counted. Experiments were repeated twice.

Treatments with inhibitors

Emetine (Sigma), stored at -20°C and at 1 mM in autoclaved distilled water, was used at three concentrations (0,1 µM; 0,3 µM and 1 µM) to inhibit translation activity. Thioluthin (Sigma), stored at 1mM in DMSO, was used at three concentrations (0,03 µM; 0,1 µM and 0,3 µM) to inhibit transcription activity. For each condition inhibitors were added before the release of the gametes from the plurilocular sporangia. This procedure ensured that the inhibitor was present very early and gave time for the inhibitor to act at the very early stages. Gametes were allowed to settle on glass cover-slips and the medium was changed every two days. For the analysis of recovery, inhibitors were removed by washing the partheno-sporophytes (pSP) three times with PES, and then cultivating them in fresh medium for up to 14 days.

The same proportion of DMSO was included in the controls. Treatments were continuous, and medium with inhibitor was changed every two days. For the recovery studies, partheno-sporophytes were washed three times in PES and allowed to develop in PES.

Microscopy

Fluorescent Brightener 28 (Calcofluor, Sigma) was diluted at 1 mg.mL-1 in autoclaved distil water, filtered at 0.2 µm and stored at -20°C. To stain cell-wall calcofluor stock solution was 100-fold diluted in PES, added and incubated for 15 minutes to day 14 germinated gametes, and washed three times with PES (Excitation at 365nm and emission at 435nm).

Germinated gametes at day 5 and day 12 were fixed overnight using glutaraldehyde at 1% final (diluted in PES). Fixation was followed by a DNA staining using DAPI. DAPI was diluted in 1% autoclaved PBS, added for 30 min to fixe germinated gametes and washed three times using 1% autoclaved PBS.

After 12 days of development with emetine, plasmic membrane was stain with the 12% of vital dye FM-64 (Sigma).

III. Results

Transcription inhibitors do not affect germination nor the first cell division but do prevent further development of *Ectocarpus* partheno-sporophytes

To evaluate the role of transcription in the regulation of parthenogenetic development, we monitored the effect of the transcription inhibitor thiolutin, on the early parthenogenetic development of *Ectocarpus* gametes. The inhibitor was applied very early, before the gametes were released from the plurilocular gametangia, and the inhibitor solution was refreshed every two days. Development of partheno-sporophytes was followed up to 12 days after release of the gametes. For each concentration of thiolutine, germination of the gametes proceeded with no significant difference compared with the control (Two-ways ANOVA followed by a Bonferroni post-tests; p-value < 0.01 for each day). The first cell division was slightly delayed, although not significantly (Two-way ANOVA followed by a Bonferroni post-test; p-value > 0.05 at day 5). Thiolutin, therefore, did not affect development of the partheno-sporophytes up to the 5-cell stage (Figure 11-B). However, continuous incubation affected further cell divisions. After 12 days 66% of the partheno-sporophytes were blocked at the 6-10 cell stage, while 92% of the control filaments had more than 10 cells.

Thiolutin induced a delay in the development in a dose-dependent manner (Figure 11-A). Inhibition of transcription did not have an effect on the overall pattern of development of

the young partheno-sporophytes and cells of the treated samples resembled non-treated partheno-sporophytes (Figure 11-C). No toxicity was observed at the concentrations tested and the effect of thiolutin was reversible. When the inhibitor was washed out at 12d AR, and development was followed for 14 days the treated partheno-sporophytes recovered and exhibited a normal pattern of development at later stages, becoming fully functional (producing upright filaments and plurilocular sporangia) and showing no difference in morphology compared with control samples (Figure 11-C).

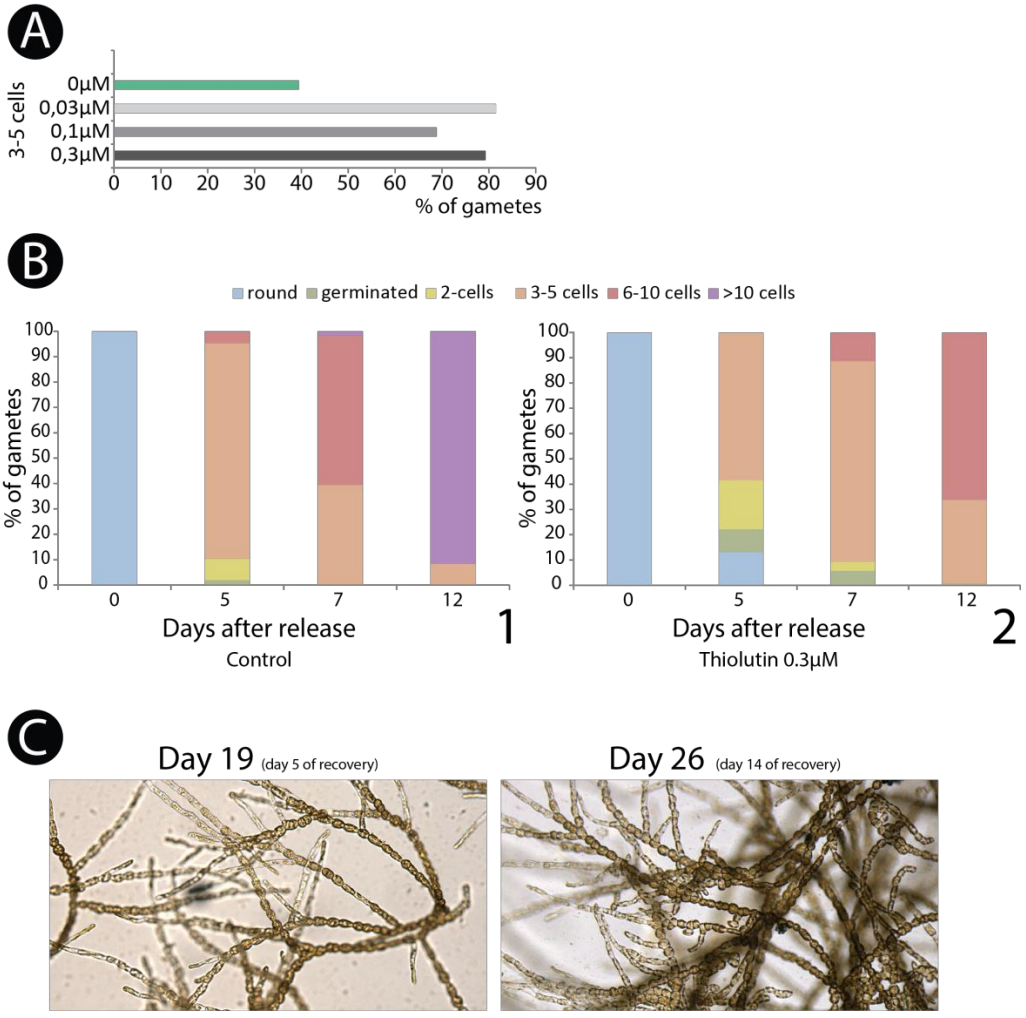


Figure 11. Effect of thiolutin on *Ectocarpus* parthenogenic development.
(**A**) Dose dependent inhibition of parthenogenic development by thiolutin at 7 days after release of the gametes from the plurilocular gametangia. Freshly released gametes were incubated with various concentrations of thiolutin for 12 days. Germination, cell division, and further development were scored at five, seven and 12 days after the beginning of the treatment. At least 100 developing partheno-sporophytes were scored. The graph is representative of three independent experiments. (**B**) Development of *Ectocarpus* partheno-sporophytes in standard culture conditions **(1)** and in 0.3µM of thiolutin **(2)**. Early development of partheno-sporophytes was in six categories (round gametes, germinated, two-cell stage, 3-5 cells stage 6-10 cells stage and >10 cells stage). Individuals were scored immediately after settlement of the gametes, and during several days after the beginning of the parthenogenesis: five, seven and 12 days. The graph is representative of three independent experiments. (**C**) Partheno-sporophytes that had been previously incubated in thiolutin (0.3µM) for 12 days and then in NSW for day 19 and 26.

Emetine prevents the first cell division in *Ectocarpus* partheno-sporophytes

We investigated the effect of an inhibitor of translation on the early development of *Ectocarpus* partheno-sporophytes. Gametes were released into seawater containing the emetine to ensure inhibition at very early stages. The inhibitory effect of emetine was dose-dependent (Figure 12-A). Gamete germination was not affected by inhibition of translation, with approximately 80% of gametes having germinated after 12 days, but the first cell division was inhibited (Figure 12-B). Approximately 25% of the cells escaped inhibition and progressed to the 2-5 cell stage, but further development of treated partheno-sporophytes was strongly compromised (Figure 12-C).

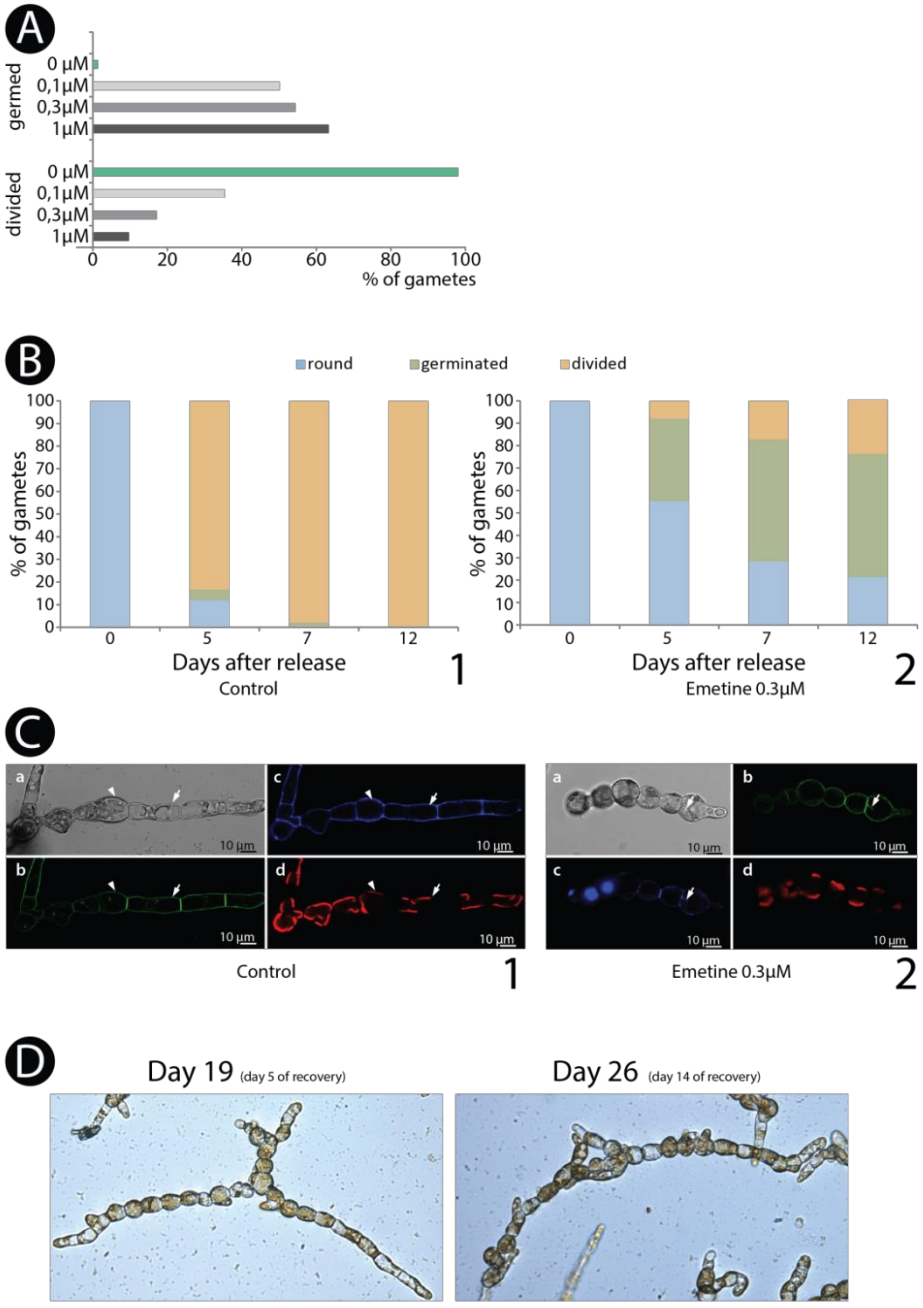


Figure 12. Effect of emetine on *Ectocarpus* parthenogenetic development.

(**A**) Dose dependent inhibition of parthenogenic development by emetine at 7 days after release of the gametes from the plurilocular gametangia. Freshly released gametes were incubated with various concentrations of emetine for 12 days. Germination, cell division, and further development were scored at five, seven and 12 days after the beginning of the treatment. At least 100 developing partheno-sporophytes were scored. The graph is representative of three independent experiments. (**B**) Development of *Ectocarpus* partheno-sporophytes in standard culture conditions (**1**) and in 0.3µM of emetine (**2**). Early development of partheno-sporophytes was in three categories (round gametes, germinated, and divided). Individuals were scored immediately after settlement of the gametes, and during several days after the beginning of the parthenogenesis: five, seven and 12 days. The graph is representative of three independent experiments. (**C**) Images of partheno-sporophyte filaments of *Ectocarpus* after 14 days in standard culture conditions (**1**) and in 0.3µM emetine (**2**). Bright field (**a**), cell wall (calcofluor white) (**b**), membrane (FM-64) (**c**) and autofluorescence (**d**). (**D**) Long term effects of emetine on parthenogenesis. Note the abnormal cell division planes and the overall disturbed pattern of development of the partheno-sporophytes, despite 26 days of recovery in a medium without inhibitor.

Early treatment with translation inhibitor results in abnormal patterning of *Ectocarpus* partheno-sporophytes

To further understand the consequences of translation inhibition on long-term patterning, partheno-sporophytes were treated with the inhibitor and then allowed to develop for several days in inhibitor-free seawater. The majority of the partheno-sporophytes regained their capacity to undergo cell division. However, the pattern of development was markedly affected by the early incubation in emetine. Despite more than 14 days of recovery, the morphology of the filaments that had been previously treated with emetine remained abnormal, with abnormal orientation of cell division planes and abnormal composition of round vs elongated cells in the main filament (Figure 12-C-1 and 12-D). In contrast to normal partheno-sporophytes, which developed upright filaments after 3 weeks in culture, early treated partheno-sporophytes did not produce upright filaments, even after 5 weeks. These results suggest that the long-term effects of emetine were either due to the inhibition of the first cell division or to the inhibition of translation at a particular developmental stage. Similar effects were obtained using another inhibitor of translation, the cycloheximide (Figure 13)

In summary, inhibition of transcription affected cell divisions after the 6-10 cell stage, while inhibition of translation immediately inhibited the first cell division. Neither inhibitor had any effect on gamete germination (Figure 13 summary of effects).

To determine whether the long-term effect of treatment with emetine and cycloheximide was due specifically to the inhibition of protein synthesis at a particular stage of development or if it was a more general effect of blocking the first cell division, we tested other inhibitors that are known to block germination/cell division in brown algae. Nocodazole inhibits microtubule polymerisation. Continuous incubation in nocodazole inhibited the first cell division, but not germination (Figure 13). As observed in the long-term effect of emetine, nocodazole has a long-term effect on the later developmental pattern (not shown). We conclude that the long-term effect of the inhibition of protein synthesis was possibly due to the blocking of the first cell division and not specifically to inhibition of protein synthesis.

Inhibitor	Effect	Range of concentrations used	Effect on germination	Effect on 1st cell division	Effect after 5th cell division	Long term effect (after recovery)
Emetine	Translation	0,1-0,3-1µM	no	yes	yes	Cell morphology
Thioluthin	Transcription	0,03-0,1-0,3µM	no	no	yes	no
Nocodazole	Tubulin	1 µg/ml-10 µg/ml	no	yes	yes	Cell morphology
Cycloheximide	Protein synthesis	0.01, 0.05, 0.1, 0.5 µg/ml	no	yes	yes	Cell morphology

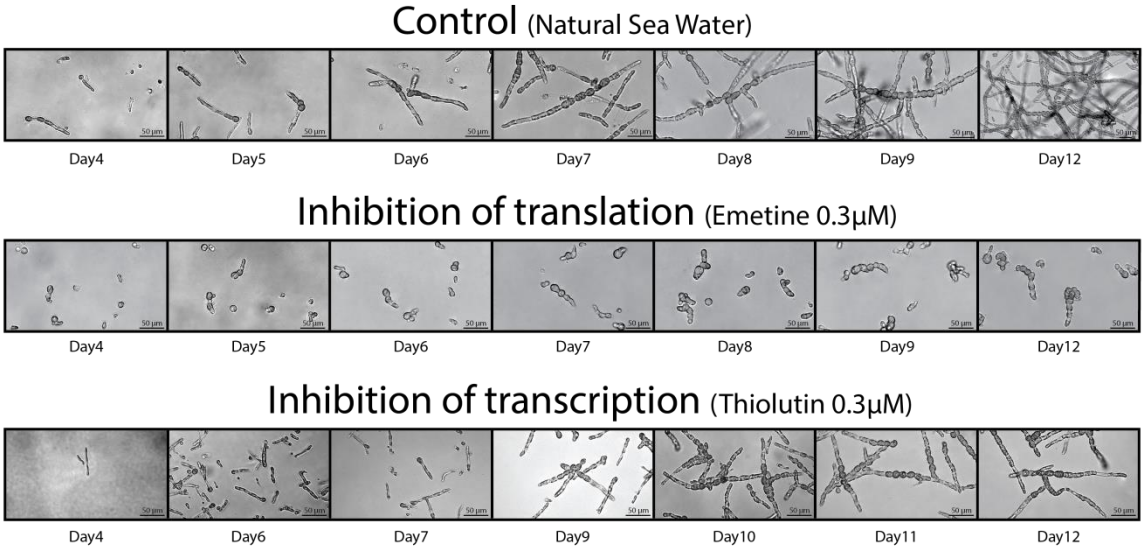


Figure 13. Effects of transcription and translation inhibition during parthenogenic development of *Ectocarpus*. (A) Table summarizing the effects of early inhibition of transcription and translation on the development pattern of the partheno-sporophytes. (B) Bright field images illustrating the development of partheno-sporophytes at different developmental stages in control samples (upper) and in presence of thiolutin (middle) and emetine (bottom).

IV. Discussion and perspectives

While an important amount of work has been published in the early stages of development of zygotes, less is known about the mechanisms regulating parthenogenetic development. *Ectocarpus* gametes can develop parthenogenetically if they do not meet a partner of the opposite sex (Bothwell *et al*., 2010). We analysed the early development of *Ectocarpus* partheno-sporophytes under control conditions and in the presence of inhibitors of transcription and translation. These analyses showed that germination of non-fused gametes is independent of *de novo* transcription and translation, strongly suggesting that gametes use their existing protein stocks during the first steps of parthenogenetic development.

The first cell cycle does not require *de novo* transcription, but does require translation activity, suggesting that messenger mRNAs encoding for proteins involved in progression through the cell cycle are already present in the gamete. Recent work which described the transcriptome of *Ectocarpus* gametes (RNAseq) supports this hypothesis (Lipinska *et al*., 2013). Surprisingly *Ectocarpus* gametes contain transcripts for an important proportion of the genes present in the genome despite their relative small size. Specifically, mRNAs that code for cell cycle proteins are present in male gametes (Lipinska *et al*., 2013). These cell cycle proteins include mitotic kinases such as CDK1, NEK and Aurora-like kinases (Esi0053_0199, Esi0010_0208, Esi0027_0155), cyclins involved in G1/S and G2/M transition of cell cycle (cyclinD3 Esi0176_0001; Cyclin A Esi0148_0011; cyclin B Esi0071_0052), and Smc4, a subunit of condensin, a complex involved in chromosome assembly and segregation in mitosis (Figure 13). Interestingly, it has been shown that the early development of the *Fucus* zygote depends on translation of a CDK kinase mRNA, translation triggered by fertilization (Corellou *et al*., 2001).Whether this is also the case in *Ectocarpus* remains unknown.

Transcripts related to transcription and translation are among the 100 mostly expressed genes in gametes (Lipinska *et al*., 2013). mRNAs for protein metabolic processes, in particular biosynthetic pathways (ribosome and translation related) are also present in gametes. Without *de novo* transcription, partheno-sporophytes can proceed through 5 successive cell divisions. These data suggest that mRNAs stocks present in the gametes are sufficient to support growth, cell division and metabolic processes necessary for the first steps of development of the partheno-sporophyte.

Continuous incubation of *Ectocarpus* partheno-sporophytes with emetine inhibited the first cell division. In the long term, however, although partheno-sporophytes recovered from

the treatment and cell divisions proceeded, they developed into abnormal partheno-sporophytes affected in their developmental patterning. In many multicellular organisms the early-stages of zygote development are critical to the determination of different cell fates of the early embryonic cells (Brownlee and Bouget, 1998; Corellou *et al*., 2000; Lin and Schiefelbein, 2001), and this seems also to be the case for *Ectocarpus*.

In the life cycle of *Ectocarpus* the development of the sporophyte occurs during sexual and asexual reproduction with zygotic development and parthenogenetic development respectively. Sporophytes produced after fusion or not of gametes share the same developmental pattern producing morphologically identical sporophytes (Peters *et al*., 2008), suggesting strongly that both zygotic and parthenogenetic sporophytes are functionally identical. Despite the similitude between *Ectocarpus* partheno-sporophytes and zygotic sporophytes there are some differences, such as the delay of germination. The sporophytic program is immediately triggered after the fusion of the two gametes while unfused gametes start the sporophytic program after approximately 24 hours. The mechanisms underlying the rapid triggering of the sporophytic development after fusion of gametes remain unclear and more work on the dependence of zygotic development on transcription and translation would help to further understand the role of these processes during sporophytic development.

Use of inhibitors that block the translation, such as the emetine and cycloheximide, affected the first cell division but also modified the developmental pattern of the partheno-sporophyte in the long-term. This long-term effect was also observed using a more specific inhibitor, nocodazole that inhibits the polymerization of microtubules. Therefore, the long-term effect of emetine and cycloheximide were probably more related to the fact that the first cell division was affected than to the inhibition of translation *per se*.

Chapter 7. General Conclusions and Perspectives

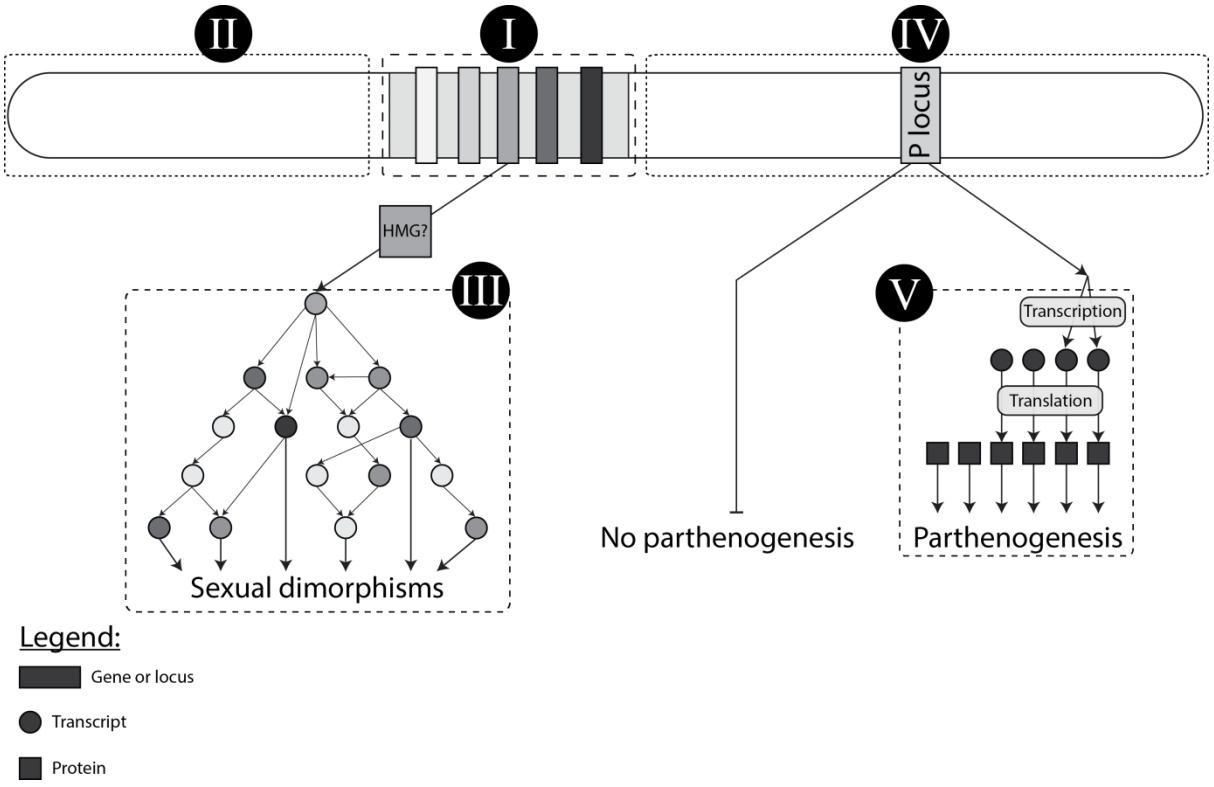


Figure 14. Global view of the PhD work.

I. Identification of *Ectocarpus* UV sex chromosomes. The first chapter of the thesis reported the characterization of male and female SDR. UV sex chromosomes are diverging for more than 70 Mya. As predicted by Bull (1978), the haploid purifying selection probably limited the genetic degeneration of *Ectocarpus*' SDR.

II. To go further on the characterization of UV sex chromosomes, the unusual genomic features of PAR were analysed. Both empirical and theoretical modelling provided evidence for an enrichment of generation biased genes on the PAR, provided that these have different selection pressures in males and females.. From this study two working hypotheses were elaborated to explain the evolution of the *Ectocarpus* UV sex chromosome system.

III. Genes downstream of the SDR master sex determining gene(s) are responsible for the phenotypic differentiation between male and female developmental programs. In this study we showed that *Ectocarpus* exhibits a low level of sexual dimorphisms coherent with the low abundance of SBG found in *Ectocarpus*, and that the evolution of SBG is different to other systems described so far.

IV. The genetic basis of a sexual dimorphic trait (parthenogenesis) was explored in this thesis. This analysis pointed out that the parthenogenesis locus is located on PAR of *E.siliculosus*. We have shown that this parthenogenetic locus is possibly under sexual antagonistic selection.

V. We used cell biology approaches to understand the cellular basis of parthenogenesis in *Ectocarpus*. This study showed that the germination of gametes is independent of transcription and translation and that parental transcripts and proteins contained in the gametes are sufficient for the first five cell divisions of the partheno-sporophyte.

This PhD thesis has made an important contribution to increase our knowledge on the evolutionary history of UV sex chromosomes and has helped to elucidate how the sex chromosome of the model brown alga *Ectocarpus* mediates sexual differentiation. *Ectocarpus* belongs to the brown algae, a major eukaryotic lineage very distantly related to animals and plants, that has been largely understudied. Elucidation of the mechanisms of sex determination in a brown alga represented therefore an important opportunity to test hypotheses about the evolutionary dynamics of sex-determining systems in a broad evolutionary context. Accordingly, we showed that the *Ectocarpus* UV has had a distinct evolutionary trajectory to the well-studied XY and ZW systems, although some striking similarities were revealed, indicating the remarkable universality of some of the underlying processes shaping sex chromosome evolution across extremely distant lineages.

The *Ectocarpus* non-recombining region has evolved more than 70, possibly more than 100 Mya, but despite its age it exhibited a low level of genetic degeneration and has remained relatively small. Expression analysis of sex-linked genes during the life cycle of *Ectocarpus* indicated, as predicted by Bull (1978), that UV sex chromosomes experience haploid purifying selection, limiting the genetic degeneration. The same analysis has identified several genes specifically expressed during the male maturity and therefore probably having an important role in the male sex-determination pathway. Among those genes the male-specific HMG gene (*Esi0068_0016*) is a strong candidate for triggering the male-determining pathway, and therefore deserves to be further analysed. Therefore, future work should focus on the validation and functional analysis of the *Esi0068_0016* gene. First, the role of the male-specific HMG protein in the male-determination pathway needs to be tested, ideally by specifically knocking-out this gene or to inhibit its expression. Genetic transformation is under development for *Ectocarpus*, and RNAi silencing using dsRNA for the HMG-gene is currently being trialled. In parallel, a search for sex-reversed mutants is being carried out, together with a screen of a TILLING mutant collection. If its role is confirmed, this will evoke important questions concerning the evolution of sex-determination gene cascades across the eukaryotes. HMG proteins are transcription factors that bind to specific DNA sequences to regulate the expression of targeted genes (Bianchi and Agresti, 2005). It would be interesting to use a ChIP-Seq approach (Robertson *et al*., 2007) to characterise binding sites of the *Ectocarpus* male-specific HMG protein in order to identify its direct targets and access the genes involved in the male-determining cascade. Currently, the

Algal Genetics Group is already using a pull-down approach (Einarson *et al*., 2007) to identify proteins that interact with the Esi0068_0016 HMG protein.

Genes differentially expressed between sexes play an important role in the establishment and maintenance of the differences that characterize female and male. Such genes in *Ectocarpus* were analysed and analysis showed that only about 12% of the expressed genes were differentially expressed between sexes. This low level of differential gene expression reflects the low level of phenotypic sexual dimorphism, which possibly indicates a low intensity of sexual selection, leading to less scope for SA selection to favour sex-biased expression to resolve sexual antagonism. In this context it will be interesting to take advantage of the broad range of sexual dimorphisms found in brown algae to analyse the abundance of SBG in diverse sexual dimorphic species of this group. Such an analysis would indicate to what extent expressed genes have sex-biased patterns in brown algae and would allow correlation between the abundance of SBG and the level of sexual dimorphism to be studied. These two parameters have been shown to be positively correlated in turkey (Pointer *et al*., 2013). Such a broad analysis of brown alga transcriptomes would provide a better understanding of the evolution of sexual dimorphism in brown algae, but would also allow the identification of sex-linked genes in others species. More information about genes that are part of SDR in other brown alga would be valuable to have a full scale overview of the evolutionary dynamic of sex chromosomes in the brown algae group.

During this thesis, the *Ectocarpus* sex chromosomes were further characterized by analysing the pseudoautosomal regions (PARs). Work on the *Ectocarpus* PAR showed that this genomic region has many unusual features and has led to two hypotheses for the evolution of UV sex chromosomes in brown algae: the “SDR contraction” hypothesis where UV sex chromosomes could have restored their capacity to recombine and keep a smaller non-recombining regions and the “SDR expansion” hypothesis where SDR evolved by SA selection).

Interestingly the finding of an ortholog of *Ectocarpus* (Ectocarpales) PAR gene, *Esi0285_0026*, sex-linked in *Undaria* (Laminariales), could provide some interesting indications concerning the evolutionary history of UV sex chromosomes in brown alga. It would be interesting to be able to date when this gene became part of the *Undaria* SDR, and to determine the divergence of male and female alleles of the gene. Such information could probably resolve between the “SDR expansion” and “SDR contraction” hypotheses for the evolution of SDR in brown alga. A recent insertion in the *Undaria* SDR or a low level of

divergence between female and male version of this gene in *Undaria*, would indicate that sex chromosomes in brown algae evolve by expansion, through SA gene capture. On the contrary if the male and female alleles of the *Undaria* orthologue of *Esi0285_0026* are highly divergent, it would indicate that the SDR evolved under the "SDR contraction" hypothesis. Such an evolution of sex chromosomes in brown algae would be consistent with the phylogeny of brown algae that suggests that the common ancestor of all brown algae but also to Laminariales and Ectocarpales was oogamous (Silberfeld *et al*., 2010). Indeed if the common ancestor of Laminariales and Ectocarpales was oogamous the diminution of sexual dimorphism in Ectocarpales (all Ectocarpales are isogamous or anisogamous; Silberfeld *et al*., 2010) may have been accompanied by a restoration of recombination that led to the shrinking of the SDR in Ectocarpales (Figure 15). Another way to understand the evolutionary dynamic of those sex chromosomes, and therefore to test the two previous hypotheses mentioned, would be to sequence and characterize sex chromosomes in other brown algae.

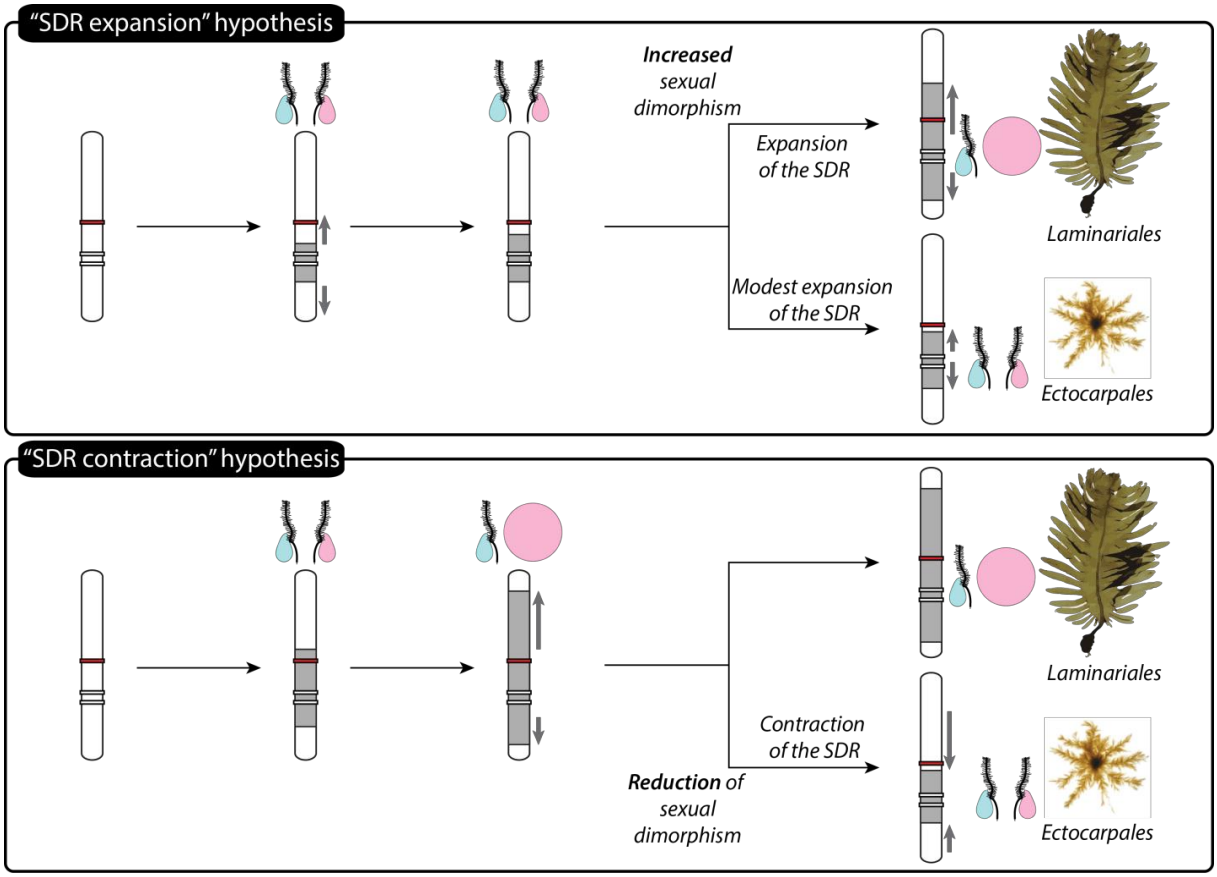


Figure 15. Hypotheses for the evolution of UV sex chromosomes in brown algae.

Under the **"SDR expansion" hypothesis** the non-recombining SDR evolved through sexual antagonistic (SA) forces. The higher level of sexual dimorphism in Laminariales compare to Ectocarpales probably come along with higher SA forces and led to the integration of the *Ectocarpus'* orthologous gene *Esi0285_0026* (red line) in *Undaria* (Laminariales).

Characterization of the *Ectocarpus'* PAR (see Chapter 3) has led to an alternative hypothesis: the **"SDR contraction" hypothesis**. The latter could explain the evolution of UV sex chromosomes by a reduction of the level of sexual dimorphisms in the Ectocarpales accompanied by a restoration of recombination and therefore a contraction of the SDR. Such a restitution of recombination capacity would probably leave some footprints of the non-recombining history, as it was observed in the *Ectocarpus'* PAR. The maintenance of the level of sexual dimorphism in Laminariales would probably allow to keep a bigger non-recombining region. This "SDR contraction" hypothesis is consistent with the brown algae phylogeny which indicate that the common ancestor of Laminariales and Ectocarpales was oogamous (Silberfeld *et al*., 2010).

To understand the potential of *Ectocarpus* SDR to expand we could analyse the level of sexual antagonism in U and V chromosomes, by searching for signatures of SA selection such as high nucleotide diversity and balancing selection (Qiu *et al*., 2013).

One of the objectives of this PhD was to identify and characterize the genetic basis of parthenogenesis in *Ectocarpus siliculosus*. Parthenogenesis can be a sexual dimorphic trait in some populations or species of *Ectocarpus*: female gametes are able to perform parthenogenesis but males are not. We have shown that this sexual dimorphic trait is determined, at least in part, by a locus located in the recombining regions of the sex

chromosomes (PAR). In the scope of this thesis, the locus controlling the parthenogenesis locus could not be finely mapped. The use of SHOREmap was however usefull to provide clues about the genomic location of the parthenogenetic locus, but it is probably more appropriate to use this technique for strains of *Ectocarpus* for which a reference genome is available. The data generated by the SHOREmap can be used to perform a *de novo* genome assembly in order to identify sequences corresponding to the *E. siliculosus* UV sex chromosomes and possibly explore new candidate regions for the identification of parthenogenesis locus. Once the parthenogenetic locus is identified, an association genetics approach could be used to confirm the identification of the locus, involving associating P+ and P- phenotypes with P+ and P- genotypes in several field populations of *Ectocarpus*.

During the analysis of the parthenogenetic locus a new interesting hypothesis appeared and will need some further tests. In another Ectocarpales species, *Scytosiphon lomentaria*, the absence of parthenogenesis in male was hypothesized to originate from mitochondrial disappearance (Han *et al*., 2014). The microscopic analysis of the integrity of mitochondria in young P+ and P- males of *E. siliculosus* was initiated but need to be continued to test if the integrity of mitochondria is important for the parthenogenetic development. The fitness analysis of P- and P+ males identified the parthenogenetic locus as a potential SA locus, with the allele P- being advantageous and P+ harmful when found in males. The fitness effects were found at the level of the zygotes, which can be coherent with the "mitochondrial" hypothesis. Indeed, under this hypothesis males' capacity to do parthenogenesis depend on the disappearance or not of mitochondria. If males P+ lose the capacity to remove mitochondria, a cross with a female will generate biparental inheritance of mitochondria in zygotes. Such a situation probably would generate cytoplasmic conflict and therefore decrease the zygotic fitness, as observed in our study. Furthermore this hypothesis is easily testable by comparing the mitochondria content of zygotes produced with P+ and P- males. As in Peters *et al*. (2004a) crosses have to be performed between two strains whose organelles are genetically distinguishable. P+ and P- males can be cross with a Peruvian female (known to be polymorphic) and the origin of mitochondria in zygotes can be follow by using specific markers to maternal and paternal mitochondrial DNA.

Interestingly, the fitness analysis of P- and P+ males in *Ectocarpus* indicated that the parthenogenetic locus may be a SA locus, with the allele P- being advantageous and P+ harmful for zygotic sporophytes, when found in males. Fitness effect on zygotes is coherent with the "mitochondrial" hypothesis. Indeed, under this hypothesis the males' capacity to

undergo parthenogenesis depends on the presence of mitochondria. If P+ males lose the capacity to remove mitochondria, a cross with a female will generate biparental inheritance of mitochondria in zygotes. Such a situation could generate cytoplasmic conflict and therefore decrease zygotic fitness. This hypothesis could be tested by comparing the mitochondrial content of zygotes produced with P+ and P- males. As in Peters *et al*. (2004a) crosses have to be performed between two strains whose organelles are genetically distinguishable. P+ and P- males could be crossed with a Peruvian female (known to be polymorphic) and the origin of mitochondria in zygotes could be followed by using specific markers of maternal and paternal mitochondrial DNA.

The study of the effects of translation and transcription inhibitors on parthenogenetic development showed that germination occurs independently of translation and that parthenogenetic development is independent of transcription up to approximately the fifth cell division. Interestingly the phenotype observed following inhibition of transcription strongly resembled the P- phenotype, which suggest that the P- phenotype could be due to a lack of transcription. Therefore, we can hypothesize that the non-parthenogenetic strains are affected in a key gene for the parthenogenesis development, gene that need to be transcribe after five cells division: the “mutation” hypothesis. Of course the “mitochondrial” and the “mutation” hypotheses to explain the non-parthenogenetic phenotype can be part of a unique hypothesis, with a mutated gene involved in the mitochondrial inheritance.

In conclusion, the study of *Ectocarpus* during this PhD has allowed to increase our understanding of the evolution of haploid UV sex chromosomes and sexual dimorphisms. The use of new sequencing technologies in brown algae will significantly increase our knowledge in the biology of this group, despite the fact that some technical limits remain (e.g. reverse genetic tools such as transformation are still unavailable). Current efforts aim at circumventing those technical difficulties, and hopefully the brown algae will continue to provide us with some very exciting discoveries.

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Annexes

Annexe 1: Sexual dimorphism in the brown algae paper

Title:

Sexual dimorphism in the brown algae

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Article

Sexual dimorphism in the brown algae

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With 3 figures and 1 table in the text and appendix

Abstract: Sexual dimorphisms have been described in several groups of organisms, but while an important number of investigations have focused on animal and plant systems, much less is known about this phenomena in other eukaryotes. We review here the current knowledge on sexual dimorphisms in the brown algae, a group of multicellular eukaryotes that have been evolving separately from animals and plants for more than a billion years. We discuss the ecological implications of these sexual dimorphisms, describe recent studies aimed at understanding the molecular basis of sex-related differences, and highlight the advantages of the brown algae to study the evolution of sexual dimorphism in a broad evolutionary context.

Keywords: sex, seaweed, evolution, sex chromosomes, isogamy, anisogamy, gamete size

Introduction

Sexual dimorphisms, which can be defined as phenotypic differences between male and female individuals of the same species, have been described to various degrees in many different groups of eukaryotic organisms. In his book on sexual selection Darwin (1871) described many examples where females and males within a single animal species differed dramatically in morphology, colouration, size, and behaviour. He proposed that gender-related differences evolved due to sexual selection resulting from variation in mating success among individuals. In recent years, there has also been a growing interest in plant sexual dimorphism (e.g. Delph et al. 2010, reviewed in Barrett & Hough 2013).

The aim of this short review is to discuss what is currently known about sexual dimorphism in brown algae, a group of multicellular eukaryotes that has evolved independently from animals and plants for more than a billion years, and to explore the potential of this group as a source of alternative model systems to study this phenomenon. We discuss the sexually dimorphic traits that have been identified in brown algae and some of the ecological implications of these dimorphisms. We also look at recent work aimed at investigating the molecular basis of sex-related differences in this group.

The brown algae exhibit a broad range of differences between male and female gametes, including isogamous (gametes of the same size), anisogamous (where the female gamete is larger than the male gamete) and oogamous species (where the female gamete is larger and non-motile). Classically, males and females are defined based on the relative size of the gametes they produce, females producing relatively few, large and usually non-motile gametes (eggs or ovules) and males producing many, small and often motile gametes (sperm or pollen). For the purpose of this review we will use the terms "male" and "female" as employed in the phycology literature, i.e. females are defined as either producing larger gametes or, in the case of morphologically isogamous species, producing gametes that quickly settle and release a pheromone to attract male gametes. Males are defined as producing smaller gametes or gametes that swim for longer, have an exploratory behaviour and respond to the female pheromone (Berthold 1881; Maier 1995). In this context, the term "isogamy" relates strictly to the gamete size, and does not take into account the physiological and behavioural differences that are consistently present in all brown algal "isogamous" lineages.

Dioicy is prevalent in the brown algae

Sexual dimorphism can only be expressed at the level of the whole thallus in species where males and females are separate individuals. Separate males and females can occur

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either during the diploid or during the haploid phase of the life cycle, in which case the species is described as either *dioecious* or *dioicous*, respectively (see App. 1). A survey of representative species from all the main orders of the brown algae suggests that dioicy is the prevalent reproductive system in this phylogenetic group (Fig. 1). This situation contrasts markedly with that described for flowering plants, where only about 6% of species have separate sexes and this state is viewed as an evolutionary dead-end (Richards 1986; Heilbuth et al. 2001). The rarity of dioecy in flowering plants may be related to the existence of widespread self-incompatibility systems in this group, as these systems allow species to be hermaphroditic without incurring problems related to inbreeding due to selfing. To date, there is little evidence for the existence of self-incompatibility systems in the brown algae (but see Gibson, 1994) and this may account at least in part for the observed difference in the frequency of dioicy. Other land plant groups also lack self-incompatibility, including for example gymnosperms, which are mostly *monoecious* but with a few lineages that include both monoecious and dioecious members (Givnish 1980). In mosses, more than half of the species are dioicous, the remainder being *hermaphrodite* (Wyatt & Anderson 1984).

Among gymnosperms, there is a strong correlation between the mode of reproduction (dioecy or monoecy) and the mode of pollen dispersal: monoecious species tend to be wind-dispersed and dioecious species to be animal-dispersed (Givnish 1980). Efforts have been made to identify similar factors that may influence or be related to reproduction mode in brown algae. Reproductive mode may indeed correlate with ecological factors, such as position on the shore, e.g. dioecious Fucales are preferentially found on the middle shore and hermaphrodites higher up the shoreline (Vernet & Harper 1980). Interestingly, it has been noted that monoicy is occasionally accompanied by the loss of sexual reproduction, at least under laboratory conditions (Müller & Meel 1982; Kuhlenkamp & Müller 1985).

Analysis of the distribution of sexual systems across the phylogenetic tree of the brown algae (Fig. 1) suggests that there have been several transitions between modes of reproduction during the evolution of this group. This conclusion is supported by several specific reports of transitions between dioicy/dioecy and monoicy/monoecy (Peters et al. 1997; Cánovas et al. 2011). The occurrence of sterile paraphyses in dioecious female *Fucus* was hypothesized to correspond to relics of the antheridium-bearing paraphyses (Billard et al. 2005), suggestive of a shift from monoecy to dioecy in this genus.

The prevalence of dioicy across the brown algal phylogeny suggests that this may have been the ancestral state for this group. A similar situation has been described for mosses, which are found to be extremely labile in their transitions between dioicy and hermaphroditism. Here, transitions to dioicy were found to occur at twice the rate of transitions to hermaphroditism at the genus level (McDaniel et al. 2013) and dioicy has also been proposed to be the ancestral state for this group (Wyatt 1982).

Traits distinguishing male and female sexes in dioicous and dioecious species of brown algae

Several sexually dimorphic traits have been described in brown algae (Table 1). These can be divided into two main classes: 1) differences between male and female gametes and 2) differences between the male and female gamete-producing stage of the life cycle (the gametophyte generation in species with haploid-diploid life cycles, see Appendix 1). We will treat these two classes of trait separately.

Most sex-related traits that have been described for male and female gametes are related to either the different functions of the two types of gamete or are a consequence of differences in gamete size. For example, during sexual reproduction in many brown algae, female gametes swim for only a short period of time before rapidly adhering to a substratum and starting to produce a sexual pheromone. The pheromone is detected by male gametes, which then swim towards and directly interact with the female gamete (Maier 1995). As a consequence of the different roles of the male and female gametes during this process, they exhibit marked sex-related differences in swimming behaviour, pheromone production, pheromone detection and cell-to-cell interaction.

The various isogamous, anisogamous and oogamous brown algal species represent a broad range of sex-related differences in gamete size. These size differences, which are thought to have evolved as a consequence of the different selection pressures on male and female gametes, also represent sexually dimorphic traits. Anisogamy and oogamy have arisen repeatedly across the eukaryotes and these systems are thought to have been derived from simpler isogamous mating systems in ancestral unicellular species (Parker et al. 1972; Kirk 2006). Somewhat surprisingly, it has also been proposed, based on phylogenetic reconstruction, that oogamy was the ancestral state in brown algae (Silberfeld et al. 2010). If this hypothesis is correct, it suggests that it may be possible for oogamy to evolve towards isogamy, despite the fact that transitions from oogamy towards isogamy are difficult to explain from a theoretical point of view (Togashi et al. 2012). Note, however, in this context that two examples of anisogamy in the primitive fucalean species *Notheia anomala* and the primitive laminarialean species *Akkesiphyus lubricus* suggest that oogamy may have arisen within these two orders (Kawai 1986; Gibson & Clayton 1987).

Differences in gamete size in anisogamous and oogamous brown algal species may influence other characteristics. In particular gamete size is likely to be one of the factors

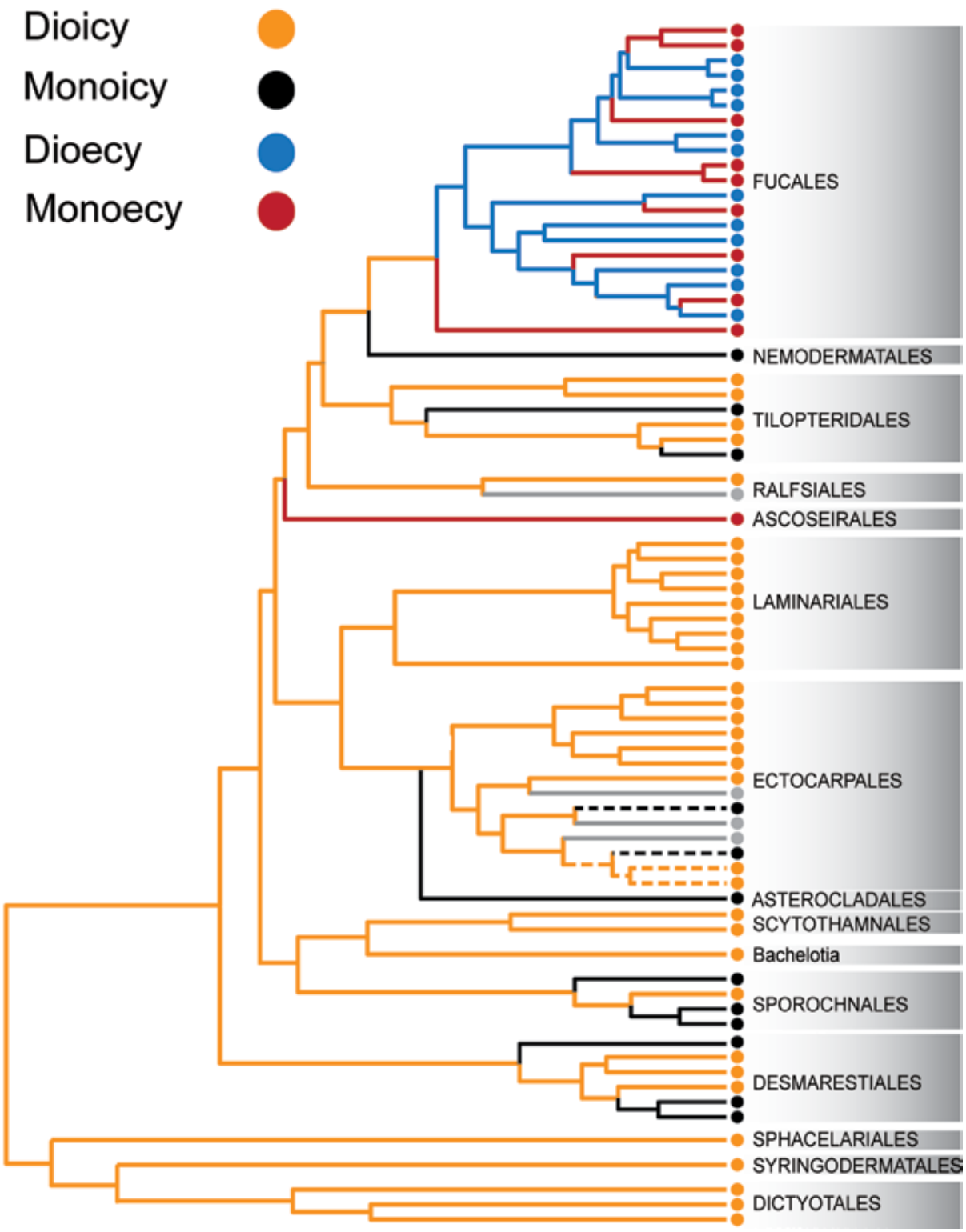


Fig. 1. Distribution of the sexual systems in the different brown algae lineages, based on the phylogenetic tree of Silberfeld et al. (2010). For simplicity, we use the terms monoicy/monoecy and dioicy/dioecy, although in some cases (some *Fucus* species for instance) the term hermaphroditism would be better adapted. The species used for this tree are the same as in Silberfeld et al. (2010) except for the following cases where species without known sexuality were replaced by closely related sexual species: *Hincksia granulosa*, *Leathesia difformis, Asperococcus bullosus, Punctaria latifolia* were replaced respectively by *Feldmannia michelliae, Chordaria linearis, Dictyosiphon foeniculaceus, Striaria attenuata*. Dashed lines were used for these species. Grey indicates lineages in which sexuality is unknown.

Table 1. Sexually dimorphic traits in brown algae. Note that gametes are considered to be parthenogenetic only if they develop into a functional individual (i.e. species whose gametes start to germinate but then degenerate were not scored as parthenogenetic). *apogamous development of sporophytes. **exceptionally yes. ***lineage according to Stache-Crain et al. (1997). [a]Most of the parthenogenetic eggs degenerate after one month. [b]A related species (*P. gracilis*) shows male and female gametophyte dimorphism. [c]A small proportion of male gametes (less than 1%) can grow parthenogenetically.

		Parthenogenesis		Gamete size		Gametophyte		Pheromone		Phototaxis		Reference
Species	**Order**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	
• *Fucus vesiculosus*	Fucales	No	No	Egg	Sperm	n.a.		Production	Attraction	Non motile	Yes	(Thuret, 1854; Overton, 1913; van den Hoek et al., 1995)
• *Notheia anomala*	Fucales	No	No	Large	Small	n.a.		*No data*		Yes	Yes	(Gibson & Clayton, 1987)
• *Nemoderma tingitanum*	Nemodermatales	*No data*	*No data*	Large	Small	n.a.		*No data*		*No data*		(Kuckuck, 1912)
• *Cutleria multifida*	Tilopteridales	Yes	No	Large	Small	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Falkenberg, 1879; Müller, 1974; Derenbach et al., 1980; van den Hoek et al., 1995)
• *Saccorhiza polyschides*	Tilopteridales	*No data*		Egg	Sperm	Larger cells; sparsely branched	Cells smaller pale; branched	Production	Gamete release and attraction	Non motile	No	(Norton, 1969; Henry, 1987b)
• *Halosiphon tomentosus*	Tilopteridales	Yes	*No data*	Egg	Sperm		n.a.	Production	Gamete release and attraction	Non motile	Yes	(Maier, 1984; Boo et al., 1999)
• *Phyllariopsis brevipes*	Tilopteridales	*No data*		Egg	Sperm	*No sexual dimorphism described*		*No data*		Non motile	*No data*	(Henry, 1987a)

Table 1 *continued*

Species	Order	Parthenogenesis		Gamete size		Gametophyte		Pheromone		Phototaxis		Reference
		Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	
• *Ascoseira mirabilis*	Ascoseirales	Yes	Yes	Isogamy	Isogamy	Gametophyte absent		Production	Attraction not found	No	No	(Clayton, 1987; Müller et al., 1990)
• *Analipus japonicus*[a]	Ralfsiales	Yes	No	Large	Small	*No sexual dimorphism described*		Production	Gamete release and attraction	Yes	Yes	(Nakamura, 1984; Müller, 1989; Nelson & De Wreede, 1989)
• *Laminaria digitata* (and all Laminariales except *Akkesiphycus)*	Laminariales	Yes	No	Egg	Sperm	Large cells; sparsely branched	Numerous and small cells; highly branched	Production	Gamete release and attraction	Non motile	No	(Sauvageau, 1918; Oppliger et al., 2011; Shan et al., 2013)
• *Akkesiphycus lubricus*	Laminariales	Yes	No**	Large, 4–5 plastids	Small, 1 plastid	*No sexual dimorphism described*		*No data*		Yes	Yes	(Kawai, 1986)
• *Pseudochorda nagaii*[b]	Laminariales	*No data*		Egg	Sperm	*No sexual dimorphism described*		*No data*		*No data*		(Kawai & Nabata, 1990; Kawai et al., 1991)
• *Chnoospora implexa*	Ectocarpales	Yes	Yes	Isogamy	Isogamy	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Kogame, 2001)
• *Colpomenia peregrina*	Ectocarpales	Yes	No	Large	Small	12–50µm; 4–8 loculi	20–40µm; 7–12 tiers of loculi (each 3–4 µm)	Production	Attraction	Yes	Yes	(Clayton, 1979; Müller et al., 1985a; Yamagishi & Kogame, 1998)
• *Petalonia fascia*	Ectocarpales	Yes	Yes	Isogamy	Isogamy	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Kogame, 1997)
• *Scytosiphon lomentaria*	Ectocarpales	Yes	Yes	Isogamy	Isogamy	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Nakamura & Tatewaki, 1975)

Table 1 *continued*

Species	Order	Parthenogenesis		Gamete size		Gametophyte		Pheromone		Phototaxis		Reference
		Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	
• *Scytosiphon canaliculatus*[c]	Ectocarpales	Yes	No	Large	Small	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	Kogame pers. commun; (Kogame, 1996)
• *Ectocarpus siliculosus*[c] *(1a***)*	Ectocarpales	Yes	No	Isogamy	Isogamy	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Berthold, 1881; Müller, 1967a; Müller, 1967b)
• *Ectocarpus siliculosus (1c***)*	Ectocarpales	Yes	Yes	Isogamy	Isogamy	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Müller, 1967b; Müller, 1967a; Bothwell et al., 2010)
• *Feldmannia mitchelliae*	Ectocarpales	Yes	No	Large	Small	n.a.		Production	Attraction	No	No	(Müller, 1969)
• *Chordaria linearis*	Ectocarpales	Yes	Yes	Isogamy	Isogamy	n.a.		Production	Attraction	No	No	(Peters, 1992a)
• *Dictyosiphon foeniculaceus*	Ectocarpales	Yes	Yes	Isogamy	Isogamy	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Peters & Müller, 1985; Peters, 1992b)
• *Striaria attenuata*	Ectocarpales	Yes	Yes	Isogamy	Isogamy	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Peters et al., 2004)
• *Scytothamnus australis*	Scytothamnales	Yes	Yes	Isogamy	Isogamy	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Clayton, 1986)
• *Splachnidium rugosum*	Scytothamnales	Yes	No	Large	Small	*No sexual dimorphism described*		*No data*		*No data*		(Clayton, 1991)
• *Himantothallus grandifolius*	Desmarestiales	Yes	No	Egg	Sperm	Large cells	Small cells	Production	Gamete release and attraction	Non motile	No	(Wiencke & Clayton, 1990)
• *Perithalia caudata*	Sporochnales	Yes	No*	Egg	Sperm	Large cells	Small cells	Production	Gamete release and attraction	Non motile	No	(Müller et al., 1985b)

Table 1 *continued*

		Parthenogenesis		Gamete size		Gametophyte		Pheromone		Phototaxis		Reference
Species	**Order**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	
• *Desmarestia aculeata (and other dioicous* Desmarestiales*)*	Desmarestiales	Yes	No	Egg	Sperm	Large cells	Small cells	Production	Gamete release and attraction	Non motile	No	(Schreiber, 1932)
• *Desmarestia firma*	Desmarestiales	Yes	No**	Egg	Sperm	Large cells, strongly pigmented	Small cells, poorly pigmented	Production	Gamete release and attraction	Non motile	No	(Anderson, 1982; Ramírez et al., 1986)
• *Phaeurus antarcticus*	Desmarestiales	No*	Yes	Egg	Sperm	Large cells, smaller filament	Small cells, narrower filament	Production	Gamete release and attraction	Non motile	No	(Clayton & Wiencke, 1990)
• *Syringoderma phinneyi*	Syringodermatales	Yes	Yes	Isogamy	Isogamy	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(Müller et al., 1982; Henry & Müller, 1983)
• *Sphacelaria rigidula*	Sphacelariales	Yes	No	Large	Small	*No sexual dimorphism described*		Production	Attraction	Yes	Yes	(van den Hoek & Flinterman, 1968)
• *Cladostephus spongiosus*	Sphacelariales	*No data*		Isogamy	Isogamy	n.a		Production	Attraction	Yes	Yes	(Müller et al. 1986; Gibson, 1994)
• *Dictyota dichotoma*	Dictyotales	Yes	No	Egg	Sperm	Broader and smaller interdichotomies	Narrow apical angles; short cortical cells; narrow medullary cells	Production	Attraction	Non motile	Yes	(Phillips et al., 1990; Tronholm et al., 2008)

that determines whether a gamete is capable of undergoing *parthenogenesis* should it fail to encounter a gamete of the opposite sex. In anisogamous and oogamous species this has led to differences between the parthenogenetic capacities of male and female gametes (Table 1). Usually both male and female gametes of isogamous brown algal species are capable of parthenogenesis whereas only the female gametes of anisogamous species are parthenogenetic (i.e. in the latter parthenogenesis is a sexually dimorphic trait). Exceptions to this trend do however exist, e.g. *Desmarestia* (Ramírez et al. 1986) or *Phaeurus* (Clayton & Wiencke 1990). Neither the male nor the female gametes undergo parthenogenesis in many oogamous species (especially in the Fucales), but there are notable exceptions in the Laminariales. Interestingly, flagella remnants have been observed in the egg cells of *Laminaria angustata* suggesting that the gametes of this species may be considered to represent an intermediate state between anisogamy and oogamy (Motomura & Sakai 1988). One interesting possibility that would merit further investigation is that the flagella remnants may play a role in female parthenogenesis in these species by allowing the formation of centrosomes in the unfertilised gamete. Overall, these trends suggest that gamete size influences parthenogenetic capacity up to a point, but that in oogamous species the large female gamete is specialised for zygote production and is no longer capable of initiating parthenogenetic development. Understanding the costs and benefits of these different reproductive strategies, particularly the incorporation of different degrees of parthenogenetic capacity in the sexual cycle, represents an interesting avenue for future research, both experimental and theoretical, and the brown algae would be a suitable group in which to study this phenomenon.

Microscopic dioicous gametophytes of species from the predominantly oogamous orders Laminariales, Desmarestiales, Sporochnales, and Tilopteridales usually show significant sexual dimorphism (Sauvageau 1915; Schreiber 1932; Müller et al. 1985b). Male gametophytes are composed of small cells and produce many gametes, whereas female gametophytes are composed of large cells and produce only a single or a small number of oocytes (Table 1, Fig. 2; Destombe & Oppliger 2011). These marked morphological differences allow rapid sexing of gametophyte clones in these groups. Exceptions to this general rule of relatively clear sexual dimorphism at the level of the gametophyte include the oogamous species *Phyllariopsis brevipes* (Tilopteridales; Henry 1987a) and *Pseudochorda nagaii* (Laminariales; Kawai & Nabata 1990) and the anisogamous species *Akkesiphycus lubricus* (Laminariales; Kawai, 1986), which have dioicous but monomorphic gametophytes (Table 1). In general, these three species have retained more ancestral characters, suggesting that the dimorphism was acquired independently in the different groups. Male and female gametophytes can also exhibit differences in terms of the timing of sexual maturation. Male gametophytes of the kelp *Alaria crassifolia* exhibit *proterandry*, antheridia of male gametophytes ripen after 4 days under favourable conditions, whereas females require 10 days (Nakahara & Nakamura, 1973). Interestingly, rather than releasing their gametes during the day in response to a light signal, oogamous species in the Laminariales, Desmarestiales, Sporochnales, and Tilopteridales release their eggs at night, which in turn induce the release of spermatozoids by producing pheromones (Table 1).

There have been no reports of sexual dimorphisms between male and female thalli of dioecious brown algal species (App. 1) such as the fucoids, but it may be necessary to carry out detailed morphometric analyses to verify that there are no subtle dimorphisms in these species.

Although future work may uncover additional sexually dimorphic traits in the brown algae, it is clear that neither brown algae nor land plants exhibit the complexity of sexual dimorphisms that have been observed in many animal groups. One of the hypotheses that have been put forward to explain the low level of sexual dimorphism in flowering plants is that because most dioecious lineages are relatively young, insufficient time has elapsed in order for marked sexual dimorphisms to have evolved in this group (Barrett & Hough 2013). This hypothesis is however unlikely to explain the low level of sexual dimorphism observed in brown algae (a least in terms of morphological complexity), as dioicy appears to be a relatively ancient

Fig. 2. Male and female gametophytes of *Laminaria digitata* in a laboratory culture (micrograph courtesy of Christophe Destombe). Male and female gametophytes are indicated by male and female symbols, respectively. The spindle or barrel-shaped single cells are diatoms.

characteristic of this group (Fig. 1). An alternative explanation may be derived from differences in the reproductive biology of algae and plants compared with animals. The former are immobile and interaction between the sexes is indirect. Most brown algae, for example, use broadcast spawning and the gametes meet and fuse in the seawater medium, without any further intervention of the gametophyte from which they originate, (except in cases where gametophyte fragmentation occurs; Destombe & Oppliger 2011). Reproductive success is assured by indirect measures such as releasing gametes at the optimal phase of the tide or by equipping gametes with efficient phototactic and pheromone systems (Maier 1995; Pearson 2006). The situation is similar for land plants, except that competition can occur between male gametes in species that receive pollen on a pistil (Pannell & Labouche 2013). In neither case, however, is there scope for the strong sexual selection that results from mate choice in motile animal species. In support of this hypothesis, it has been noted that among animals, and in particular invertebrate taxa, species that copulate generally exhibit significantly more marked levels of sexual dimorphism than species that broadcast their gametes (Strathmann 1990; Levitan 1998). Note, however, that there is nonetheless scope for sexual selection in brown algae on traits of importance for mating such as increased motility of the male gametes and higher pheromone production by the female gametes, even if there is no evidence of direct interaction between gametophytes.

Sex-dependent responses to environmental factors

In some cases, sexually dimorphic traits may be detectable only under specific, usually extreme, environmental conditions. It has been reported that abiotic factors can differentially influence the survival of male and female individuals, suggesting sex-dependent susceptibilities to the environment. Sex ratios can be modified by abiotic stresses such as salinity or temperature (Oppliger et al. 2011). In kelps, egg production takes place over a narrower range of conditions than antheridium production (Harries 1932), indicating different sensitivities of male and female gametophytes. Following exposure to high temperatures in culture, *Saccharina latissima* and *Laminaria digitata* produced a higher proportion of males (Cosson 1978; Lee & Brinkhuis 1988). Norton (1977) showed that female kelp gametophytes were more sensitive to extreme temperatures than male gametophytes, and correlated this effect with the geographical extent of the region within which sexual reproduction occurred. The opposite trend was observed for *Laminaria religiosa,* extreme temperatures resulting in a decrease in the proportion of males (Funano 1983). More recently, Nelson (2005) demonstrated that high temperature and long days resulted in a sex ratio biased toward females in *Lessonia variegata*, suggesting, again, that males were less resistant to stressful conditions. Taken together, these results suggest that the effect of temperature on sex ratio in kelps is variable and species dependent. Other factors may also affect the sex ratio, for example male and female *Saccorhiza polyschides* gametophytes showed differential sensitivities to changes in salinity (Norton & South 1969).

It is also possible that males and females respond differently to biotic factors but the limited data currently available argue against such an effect. Male and female strains of *Ectocarpus* exhibit the same susceptibility to viral infections and no difference in resistance to the oomycete pathogen *Eurychasma* has been observed between the sexes (Claire Gachon, personal communication).

Ecology

In orders with equal numbers of *monoicous* and dioicous species, such as Desmarestiales and Sporochnales, species with smaller sporophytes and a shorter life span tend to be monoicous, whereas taxa with larger sporophytes and longer lifespan are dioicous (Peters et al. 1997). In these orders, monoicy, which allows selfing, is thus favoured in r-selected species, whereas K-selected environments favour dioicy and outbreeding. *Fucus* species adapted to more stressful environments high on the shore are hermaphrodites that exhibit frequent inbreeding, in contrast to dioecious species with obligate outcrossing in more benign habitats (Billard et al. 2010). In the Ectocarpales, however, where most species are small and follow the r strategy, only a minority of taxa with known sexuality are monoicous (e.g. 10% in Chordariaceae). Additional unknown factors may underlie other differences, suggested by the observation that there are no monoicous species in the order Laminariales while monoicy is common in the orders Sporochnales, Desmarestiales, and Tilopteridales, which resemble kelps in many other aspects of their reproductive biology.

Studies of sex ratios in meiotic offspring under standard culture conditions consistently indicate a similar proportion of males and females (Sauvageau 1918; Schreiber 1932; Cosson 1978), but relatively few reports are available about brown algal sex ratios in the field. In dioecious flowering plants, females usually expend more resources in reproduction than males, and a recurrent pattern observed in this group is the presence of male-biased sex ratios in marginal populations experiencing higher levels of environmental stress (Delph 1999). In *Lessonia* (Laminariales), sex ratios were found to be favoured towards females in the limits of the distribution area (Oppliger et al. 2012). This deviation from a 1:1 ratio at the margins of the species range could be due either to differential mortality/sensitivity to temperature between sexes or to geographic variations in the degree of parthenogenesis (asexual reproduction), as females are

often parthenogenetic and males are not (Oppliger et al. 2011). Female-biased sex ratios have also been reported for some natural populations of anisogamous species (Kitayama 1992; Yamagishi & Kogame 1998), and again a correlation between female-bias and parthenogenesis has been put forward as a possible explanation. Interestingly, a link between life cycle mode and sex ratio has been reported. Populations dominated by female *Cutleria cylindrica* individuals showed a direct type of life history (spores from unilocular sporangia give rise to new sporophytes, App. 1), whereas populations with a 1:1 sex ratio presented a heteromorphic, sexual life history, alternating between sporophyte and gametophyte generations (Yamagishi & Kogame 1998). There have also been occasional reports of isogamous species in which single field sporophytes had exclusively female offspring (e.g. Müller 1979; Peters & Müller 1986; Peters et al. 1987). As both male and female gametes of these species are parthenogenetic under laboratory conditions, it is unlikely that these populations result from female gamete parthenogenesis and further studies will be required to understand how such populations arise.

Molecular mechanisms underlying sexual dimorphism in the brown algae

Sex has been shown to be determined genetically in *Ectocarpus* sp. (Müller 1967b) and heteromorphic sex chromosomes have been reported in several kelp species (Evans 1963; Yasui 1992). More recently, a putative sex-determining region has been identified in a hybrid of *Laminaria japonica* and *Laminaria longissima* (Yang et al. 2009). There is therefore accumulating evidence that sex is genetically determined in brown algae and, consequently sexual dimorphism is ultimately under the control of a specific sex-determining region (SDR) of the genome (a sex locus or a sex chromosome). Note that, in plants, transitions to dioecy are correlated with the evolution of sex chromosomes that subsequently promote the appearance of sexually dimorphic traits (Rice 1984). Identification and characterisation of SDRs in brown algal species will not only provide important insights into the evolution of sexuality and sexual dimorphism in this group but will also provide much needed molecular markers to discriminate between male and female individuals.

Based on studies of sexually dimorphic animal and plant species (e.g. Zhang et al. 2004; Mank et al. 2007) it is likely that only a small set of the genes that determine the differences between sexes are located within the SDR (although these should include the master sex-determining gene), the majority of the downstream sex-related genes being scattered throughout the genome (Ellegren & Parsch 2007). Therefore, whilst it will be important to characterise brown algal SDRs, it is also necessary to compare gene expression between the two sexes to fully understand the genetic basis of sexual dimorphism in this group. Two recent studies have carried out analyses of this type, comparing male and female individuals of *Fucus* (Martins et al. 2013) and male and female gametes of *Ectocarpus* (Lipinska et al. 2013). A general trend that has been found in both land plants and animals is that male sex-biased genes tend to be expressed more strongly than female sex-biased genes (Zhang et al. 2004) and that this appears to be correlated with male sex-biased genes being under stronger selection (exhibiting higher *dN/dS* ratios across species). This effect is thought to be due, at least in part, to widespread *pleiotropy* of female sex-biased genes (Ellegren & Parsch 2007; Mank et al. 2007). In *Fucus vesiculosus,* male sex-biased genes also exhibited greater expression bias than female sex-biased genes compared with the vegetative background, suggesting that similar processes may be operating in brown algae (Martins et al. 2013).

An analysis of sex-biased gene expression in *Ectocarpus* gametes carried out by Lipinska et al. (2013) showed more than 25% of genes were differentially expressed, which is surprising considering that this species has been reported to be isogamous. This study suggests that there may be considerable differences between male and female gametes, even when the two are morphologically indistinguishable, and raises intriguing questions regarding our perception of sexual dimorphism.

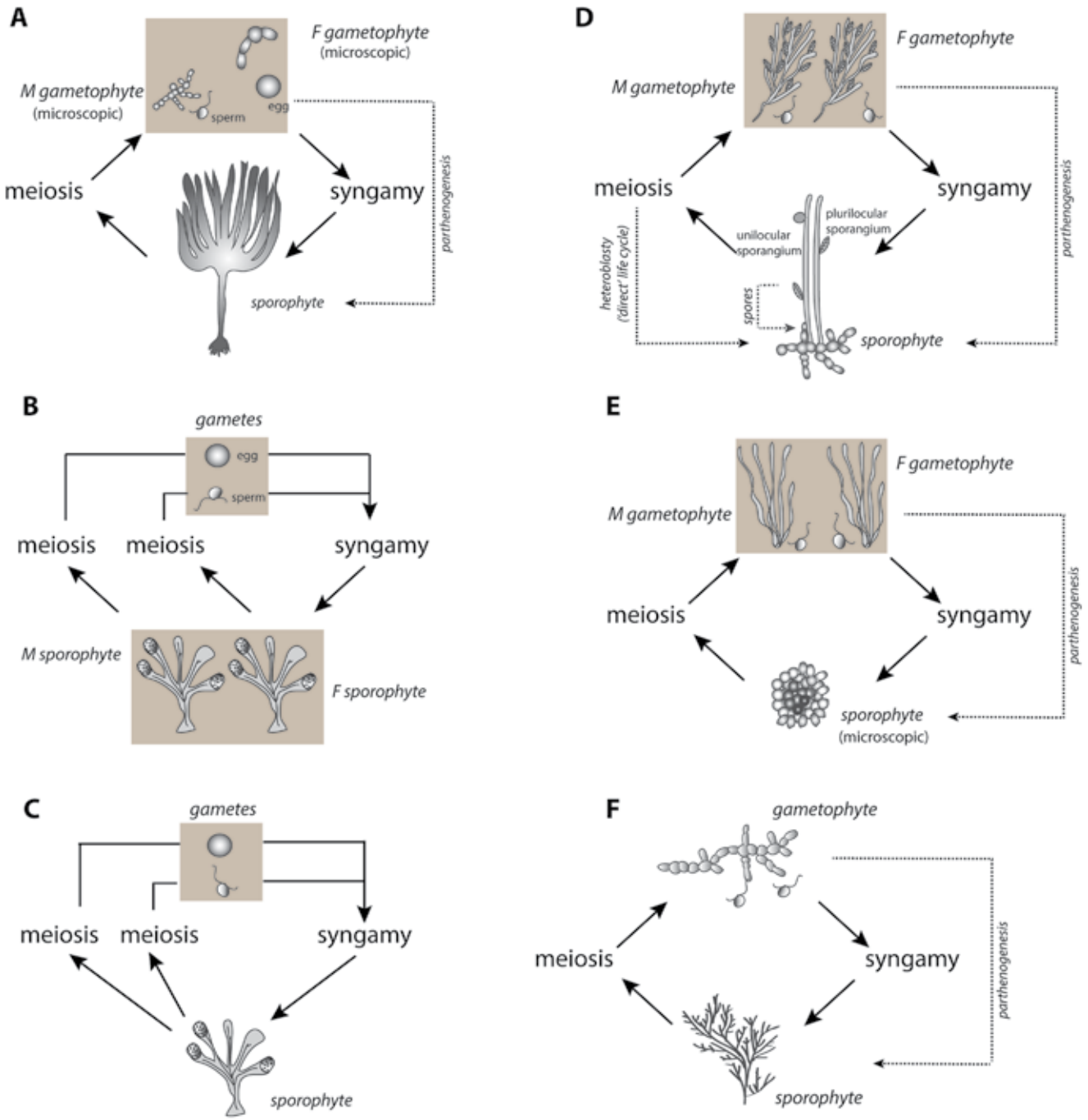
Conclusions

A number of clear sexually dimorphic traits have been described in the brown algae, observed either during the gametophyte or the gamete stage of the life cycle. In some cases these differences between male and female individuals may be important with regard to the ecology of a species, particularly at the edges of its geographical range. Despite the prevalence and probable long history of dioicy, sexual dimorphism is for most of the brown algae not as marked as in animals, possibly because the reproductive strategies of brown algae afford relatively limited scope for sexual selection. Nonetheless, the brown algae represent an interesting group for future studies of sexual dimorphism particularly with regard to gamete phenotypes as this group exhibits a broad range of gamete dimorphism from isogamous, through anisogamous, to oogamous systems. Current work aimed at identifying sex-determining regions in brown algal genomes and at comparing the transcriptomes of male and female individuals is expected to provide new insights into the molecular systems that underlie sexual dimorphisms in these seaweeds.

Appendix 1

Sexual dimorphism and brown algal life cycles

Brown algae exhibit a broad variety of life cycles, ranging from isomorphic haploid-diploid life cycles, in which both gametophyte and sporophyte generations exhibit multicellular development, to diploid life cycles, where only the diploid generation of the life cycle is multicellular (reviewed in Coelho et al. 2007; Cock et al. 2013). The ancestral brown algal sexual life cycle was presumably haploid-diploid (Silberfeld et al. 2010). In the kelps, the gametophyte generation is reduced but nonetheless develops independently of the sporophyte, and the male and female gametophytes are easily distinguishable under the microscope (**A**). In the fucoids and Ascoseira, the gametophyte generation has been lost, resulting in a diploid life cycle, with dioecious or monoecious individuals (**B** and **C**, respectively). Variations in life cycle structure occur also



within orders, for example in the Ectocarpales, which includes species with isomorphic haploid-diploid life cycles (in the Acinetosporaceae), species with slightly heteromorphic life cycles (such as *Ectocarpus*, depicted in **D**) and species with strongly heteromorphic haploid-diploid life cycles, with either the gametophyte (Chordariaceae, Adenocystaceae) or the sporophyte (Scytosiphonaceae) generation being microscopic (**E** represents an example of the latter). (**F**) Monoicous brown alga with a haploid-diploid life cycle (e.g. *Chordaria linearis*). In the figure, shaded squares represent the life cycle stages where sexual dimorphism may occur. In (**D**), *heteroblasty* refers to the development of partheno-sporophytes directly from meio-spores. M, male; F, female.

Appendix 2

Brown algae sexual systems

Brown algae exhibit a diverse range of different life cycles (Appendix 1) and this has important consequences for their sexual systems. For example, sexuality is expressed during the diploid phase in organisms with diploid life cycles such as the fucoids, whereas it is the haploid gametophyte generation that exhibits sexuality in algae such as *Ectocarpus* that have haploid-diploid life cycles (Appendix 1). Separate male and female organisms can occur in both systems but the evolutionary pathways that lead to separate sexes in each case may be very different and it is therefore important to use a nomenclature that distinguishes the two systems. The terms monoecy and dioecy are used to distinguish between species in which the diploid phase produces either both male and female gametes, on the one hand, or either male or female gametes (i.e. separate sexes), on the other. When these characteristics are observed in the haploid gametophyte generation, the terms monoicy and dioicy are used, respectively. One example of how the selection pressures that lead to the evolution of these different systems may differ is the following: whilst dioecy might evolve from monoecy to limit inbreeding (due, in the latter, to the fertilisation of female gametes by male gametes produced by the same organism), this is unlikely to be the case for dioicy because deleterious mutations should be efficiently purged during the extensive haploid phase of the life cycle. Similarly, genetic sex determination is expected to operate differently, with XX/XY or ZZ/ZW systems occurring in dioecious species but so-called U/V systems (Bachtrog et al. 2011) occurring in dioicous species.

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Glossary

Dioicous: male and female sexual structures carried separately on male and female individuals during the haploid phase of the life cycle.

Dioecious: male and female sexual structures carried separately on male and female individuals during the diploid phase of the life cycle.

dN/dS: ratio of the number of non-synonymous substitutions per non-synonymous site (dN) to the number of synonymous substitutions per synonymous site (dS), which can be used as an indicator of selective pressure acting on a protein-coding gene.

Monoicous: separate male and female reproductive structures on the same individual during the haploid phase of the life cycle.

Hermaphrodite: possessing reproductive structures that contain both male and female sexual organs.

Heteroblasty: the potential of zoids to adopt different fates.

Monoecious: separate male and female reproductive structures on the same individual during the diploid phase of the life cycle.

Parthenogenesis: development of a sporophyte or gametophyte from a non-fertilized gamete. The term parthenogenesis is classically associated with female gametes, but parthenogenesis of male gametes is common in morphologically isogamous species and male gametes of anisogamous and oogamous species may also occasionally undergo parthenogenesis.

Pleiotropy: the influence that a single gene has on multiple traits.

Proterandry: release of male gametes before the release of female gametes.

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Annexe 2: List of *Ectocarpus* strains used in this thesis.

Strain Name	Species	Lineage	Locality	Description	Generation	Ploidy	Sex locus	Study
Ec32	*Ectocarpus sp.*	1c	Peru	Male genome sequenced strain	GA; pSP	n	Male	Chapter 2; 3; 4 & 6
Ec87	*Ectocarpus sp.*	1c	Peru	Ec32 sister	GA; pSP	n	Female	Chapter 2
Ec339	*Ectocarpus sp.*	1c	Peru	SP from Ec32 x Ec25	SP	2n	Female/Male	Chapter 2
Ec588	*Ectocarpus sp.*	1c	Peru	2n homozygous GA (oro mutant)	GA	2n	Female/Male	Chapter 2
Ec602	*Ectocarpus sp.*	1c	Peru	Isogenic strain	GA	n	Female	Chapter 2 & 4
Ec603	*Ectocarpus sp.*	1c	Peru	Isogenic strain	GA	n	Male	Chapter 2 & 4
Ea1	*E.siliculosus*	1a	Naples	Parthenogenetic	GA	n	Female	Chapter 5
Rb1	*E.siliculosus*	1a	Naples	Non-parthenogenetic	GA	n	Male	Chapter 2 & 5
Ec236	*E.siliculosus*	1a	Naples	SP from Ea1 x Rb1	SP	2n	Female/Male	Chapter 5
Ec236-x	*E.siliculosus*	1a	Naples	Meiotic progeny from Ec236	GA;pSP	n	either female or male	Chapter 5

Annexe 3: List of primers used in the chapter 5. (sctg = supercontig; P locus = parthenogenesis locus)

Marker	sctg	Primer 1	Primer 1 Sequence	Primer 2	Primer 2 Sequence	Restriction enzyme	PCR product (bp)	Study
251_seq	251	251_seq_F	AACAGCTGTTAGGACACCCG	251_seq_R	GTGCCCAAGTCAAACGTAGC		581	markers to map P locus
357_caps	357	357_caps_F	TCTCCTCGACGACACTGACT	357_caps_R	CATGGATGTACGACGGCAGA	MluI	708	CAPS markers to map P locus
427_caps	427	427_caps_F	ATGGTTGTGATTCATGCGCG	427_caps_R	TGTTATCGCACGACTTCTGACA	HpaII	792	CAPS markers to map P locus
285_caps_1	285	285_caps_1_F	GGTGTATCTCCTCAGCCTGC	285_caps_1_R	CACTGCCGAAGCGTAAATCG	MluI	779	CAPS markers to map P locus
285_caps_2	285	285_caps_2_F	CGAGGTAGATAGATAGGTAG	285_caps_2_R	ACGATTGCGAGGTTCCGTAG	HpyAV	522	CAPS markers to map P locus
242_caps_1	242	242_caps_1_F	CCATGTCCTAGGTCGTGCAC	242_caps_1_R	TGGTACCTTCCGCTGTTGAA	BtsCI	727	CAPS markers to map P locus
242_caps_2	242	242_caps_2_F	CACACTCTCAACGGCACTCT	242_caps_2_R	AGATTGTGTCAACGACCGCT	BtsCI	728	CAPS markers to map P locus
242_caps_3	242	242_caps_3_F	CCTCTCGCTCTGGTTGTCTG	242_caps_3_R	CCTTCTCTCGGCGTCAAGTT	XhoI	710	CAPS markers to map P locus
105_caps	105	105_caps_F	CAATCGAAGCAATCCTGGCG	105_caps_R	TGTGATGCTGCTGTGTAGGG	PstI	667	CAPS markers to map P locus
FeScaf 02ex6	FeScaf 02	FeScaf 02ex6_F	CAGACTCGCACACATGCAAT	FeScaf 02ex6_R	GCAAGGAGATGGTCAGGAAT		200	sexing Ec236-x segregating population
FeScaf 06ex3	FeScaf 06	FeScaf 06ex3_F	CGTGGTGGACTCATTGACTG	FeScaf 06ex3_R	AGCAGGAACATGTCCCAAAC		200	sexing Ec236-x segregating population
68_25Ex4	68	68_25Ex4_F	GTCCGTATGAATGGCTGGAT	68_25Ex4_R	TTCCTTCGTGTATCGCTTGTT		200	sexing Ec236-x segregating population
68_39Ex3	68	68_39Ex3_F	AGTCAGGTCGACGCACAAG	68_39Ex3_R	GCTCCCAACAGAGGACACC		200	sexing Ec236-x segregating population

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Appendices

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Lab Positions

Since 2011 **PhD & Master degree internship**
Algal genetic team, UMR 8227
Station biologique de Roscoff, France
Supervisors: Cock JM & Coelho SM.

Genetic sex determination and evolution of sexual dimorphism in the brown algae
Aims of this project are to identify sexual dimorphisms in brown algae and to characterize their genetic basis and their evolution. The genetic basis of parthenogenesis was analyzed by using an high throughput genomic approach (ShoreMap). The evolution of sexual dimorphism in brown algae is being studied by a comparative transcriptomic approach (RNAseq).
Finaly, in order to understand the origin of those differences, the expression of male and female loci were characterized and allowed the identification of a strong candidate for the male sex-determining gene.

Mar-Jun 2010 **Research intership**
Faugeron S. team
Pontificia Universidad Catolica de Chile, Santigo
Supervisor: Faugeron S.

Physiological response of the brown alga *Lessonia nigrescens* against a thermal stress
Molecular tools such as RT-qPCR and biochemical analysis were used to identify and monitor the differential response of two criptic species of *L. nigrescens* recently identified on the chilean cost

Jan-Feb 2010 **Research intership**
Fernández M. team
The marine research laboratory of Pontificia Universidad Catolica de Chile, Las Cruces
Supervisor: Fernández M.

Identify the impact of human activity on the biodiversit by the analysis of a carnivore fish stomach contents
Identification at the level of species was performed on stomach contents of fishes on different locations: inside and outside a natural reserve. This comparative analysis was performed in order to assess the impact of fisheries

Jul-Aug 2009 **Voluntary internship**
Institute of oceanography, Villefranche s/Mer, France
Supervisors: Mousseau L, Grisoni JM & Passafiume O.

Monitoring of the coastal marine environment: SOMLIT network
Measurements of several physico-chemical parameters was performed for the french network SOMLIT (Service d'Observation en Milieu LITtoral) in order to monitor the coastal marine environement

Scientific Records

Publications

review — Cock JM, et al. (The Ectocarpus Genome consortium) (2012)
The Ectocarpus genome and brown algal genomics.
Advances in Botanical Research, vol. 64 (chapt.5): Genomic insights gained into the Diversity, Biology and Evolution of Algae
Ed. Gwenael Piganeau. Elsevier, 2012, 1st Edition.

review — **Luthringer R.**, Cormier A., Ahmed S., Peters AF., Cock JM., Coelho SM. (2014)
Sexual dimorphism in the brown algae.
Perspectives in phycology. 11-25.

article IF 9.5 — Ahmed S., Cock JM., Pessia E., **Luthringer R.**, et al. (2014)
A haploid System of Sex Setermination in the Brown Alga *Ectocarpus* sp.
Current Biology,24(17), 1945-1957.

article — **Luthringer R.**, Lipinska A., et al. (2014)
The Pseudoautosomal region of the *Ectocarpus* UV sex chromosomes.
In preparation.

article — Lipinska A., Cormier A., **Luthringer R.** et al. (2014)
Patterns of sex biased gene expression in a haploid sex determination system.
To be submitted

Meetings participation

Oral — **Luthringer R.**, Peters AF., Corre E., Lerck F., Cock JM., Coelho SM.
5th international Ectocarpus meeting.
Roscoff, France, Mars 2014. **Oral presentation**.

Oral — **Luthringer R.**, Peters AF., Ahmed S., Cock JM., Coelho SM.
Meeting of French Phycological Society.
Roscoff, France, November 2013. **Oral presentation**.

Oral — **Luthringer R.**, Peters AF., Ahmed S., Cock JM., Coelho SM.
4th international Ectocarpus meeting.
Roscoff, France, April 2012. **Oral presentation**.

Poster — **Luthringer R.**, et al.
Jacques Monod.
Roscoff, France
May 2013. **Poster**.

Poster — **Luthringer R.**, et al.
Undergraduate award for the SMBE meeting.
Kyoto, Japan
Juillet 2011. **Poster**.

Poster — **Luthringer R.**, et al.
Meeting of the French Society of Genetic.
Paris, France.
June 2011. **Poster**.

Trainings

Training 1	R introduction (may 2012)	Plateforme ABIMS (SB Roscoff)	1 Day
Training 2	R advanced (may 2012)	Plateforme ABIMS (SB Roscoff)	2 Days
Training 3	European course on comparative genomics (january - february 2013)	ENS Lyon	11 Days
Training 4	Galaxy introduction (september 2013)	Plateforme ABIMS (SB Roscoff)	1 Day
Training 5	Galaxy: RNA-seq analyses with reference genome and de novo (september 2013)	Plateforme ABIMS (SB Roscoff)	2 Days
Training 6	Galaxy: introduction to phylogeny (june 2014)	Plateforme ABIMS (SB Roscoff)	2 Days